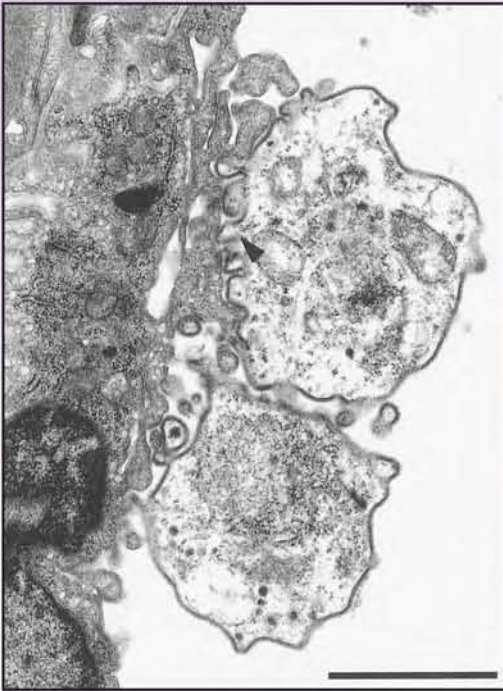


Pneumocystis Pneumonia

Third Edition, Revised
and Expanded



edited by

Peter D. Walzer

Melanie T. Cushion

PNEUMOCYSTIS PNEUMONIA

LUNG BIOLOGY IN HEALTH AND DISEASE

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the views of the National Institutes of Health.*

PNEUMOCYSTIS PNEUMONIA

Third Edition, Revised and Expanded

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In Memoriam



Professor Ann E. Wakefield 1948–2002

Ann first became interested in *Pneumocystis* in 1986, when she began work as a post-doctoral research fellow in the Department of Paediatrics, Oxford University. She rapidly developed a molecular biological approach to study the organism. By 1989 she had created the “Wakefield primers”—pAZ-102H and pAZ-102E—for amplification of DNA from *Pneumocystis*. Over the next 16 years she made an enormous contribution to our understanding of the taxonomy, molecular biology, mode of transmission and acquisition of drug resistance in *Pneumocystis*. Meeting Ann for the first time was always an experience. Her enormous drive and boundless nervous energy were tempered with tremendous humility and an infectious laugh. She possessed a razor-sharp intellect that could rapidly dissect down to the essentials of a scientific question. Ann was the quintessential research collaborator, which is reflected in the authorship of her many publications. She valued her research collaborations, not just because they furthered scientific knowledge, but also because they invariably led to enduring friendships. Her death from metastatic osteosarcoma cut short an outstanding academic career. She is greatly missed by the international “family” of *Pneumocystis* researchers.

Rob Miller
London
April 2004

Series Introduction

This is our third volume on pneumocystis pneumonia. While the disease, and some of the contributing writers, are the same as in the first (1984) and second (1994) volumes, the expanded scope of each sequential volume is indicative of ever-increasing interest and research activity in this field.

In the Preface, editors Dr. Peter Walzer and Dr. Melanie Cushion explain how this volume compares with the previous editions. It is very clear that in 20 years, the areas of investigation described in these three volumes have changed and evolved remarkably.

Pneumocystis pneumonia is still a “plague” of modern times. It is not yet conquered, but significant progress is being made and we believe we are getting closer to controlling the disease or, better yet, preventing it. While it is difficult to predict such an outcome, the odds are in our favor and, of course, the patients stand to benefit considerably.

The Lung Biology in Health and Disease series was conceived with two goals in mind. One was to report the most current state of basic and clinical research in lung disease; the other was to advance new ideas so that more research would be fostered. This volume illustrates and exemplifies these goals: it brings the reader up to date in the field, and it also stimulates new investigations.

I am thankful to the editors and the many contributors to this monograph. It is a landmark in a difficult journey toward the conquest of a worldwide public health problem.

Claude Lenfant, M.D.
Gaithersburg, Maryland, U.S.A.

Preface to Third Edition

Although the phrase, “It was 20 years ago today ...” may conjure up fond memories of a magical time for some of us in a certain generation, the 20-year time point is also a milestone in the history of this book. The year 1984 marks the publication of the first edition of *Pneumocystis carinii* Pneumonia. Dr. Lowell S. Young took on the task of editing the eight chapters that comprised the breadth of knowledge of *Pneumocystis* at that time. Peter D. Walzer, the editor of the second and now coeditor of the third edition, was the author of one of those chapters. *Pneumocystis* research was in its infancy. The causative agent of the acquired immune deficiency syndrome (AIDS) had not yet been identified, and only a handful of scientists were engaged in *Pneumocystis* research. At that time, Dr. Melanie Cushion, coeditor of the third edition, was a postdoctoral fellow in Dr. Walzer’s laboratory. Those were exciting, yet terrible times, as thousands of young men succumbed to this mysterious disease in the age of “modern medicine.” Dr. Cushion’s project was to find an in vitro culture system that would sustain the growth of rat or human *Pneumocystis* for more than a week’s time. (Little did she know the enormity of this task!) As she scrambled to understand the biology of “*Pneumocystis carinii*,” she soon realized that the history of these organisms belonged to a few dedicated investigators. Those of us who came after owe a large debt of gratitude to these early workers. This first generation of investigators provided the foundation for work in animal models; the study of immunological responses to these organisms; epidemiological concepts; and the identification of new and efficacious treatments for the often lethal pneumonia resulting from infection by *Pneumocystis*. James W. Smith and Marilyn Bartlett (Indiana University, Indianapolis, IN), Walter T. Hughes (St. Jude Children’s Research Hospital, Memphis, TN), Jack Frenkel (University of Kansas, Lenexa,

KA), Henry Masur (National Institutes of Health, Bethesda, MD), and Peter D. Walzer (University of Kentucky, University of Cincinnati, and their affiliated Veterans Affairs Medical Centers), and Julian Hopkin (Churchill Hospital, Oxford, England) were among those who labored during those early times.

The *Pneumocystis* scientific community began as a small, loosely knit group, but as the AIDS epidemic expanded, the need for additional investigators became apparent. A series of international workshops stimulated exchange among those in the field and served to gather them into a small but active community. Drs. Edna Kaneshiro (University of Cincinnati), Frank Richards (Yale University), and Peter Walzer (University of Cincinnati) initiated the first Workshop for *Pneumocystis* held in 1989 in Bristol, England. This meeting was the first to gather international scientists laboring in this new field and provided a forum for the exchange of ideas and associated problems. The first meeting and the following workshops, which continue to this day, endowed *Pneumocystis* investigators with a sense of identity and a nucleus for further growth. With them came graduate students, postdoctoral fellows, and investigators from other disciplines. Those investigators who led the charge in *Pneumocystis* research were now training the next generation of investigators dedicated to the study of these difficult, intractable, and controversial organisms. These second-generation researchers included many young scientists who are too numerous to mention here, but several contributed chapters to the second edition of this book in 1994.

Publication of this edition of *Pneumocystis* Pneumonia 10 years after the previous one is not only timely but also necessary at this juncture. The dramatic expansion of the literature on *Pneumocystis* over the past decade has resulted not only from the efforts of the first and second generations of investigators but also from the third generation, who are included in this book. The increasing volume of literature has made it difficult for many investigators in the field to keep current with the advances in the disciplines that have evolved to study these organisms. With the publication of the *P. carinii* genome in 2004/2005 and creation of microarrays using the genomic information and sequencing of the *P. jirovecii* and *P. murina* genomes by the Whitehead Institute—as well as the growing wealth of information supplied by clinical and basic research studies—these once intractable organisms will now become accessible to many more researchers, and it is anticipated that a significant number of new investigators will join the efforts to study *Pneumocystis*. The almost insurmountable impediments of the lack of in vitro culture system, reliance on chronically immunosuppressed animal models, and lack of genetic manipulation systems should now be mitigated with the creation of these new resources. We hope that this edition will serve as a one-stop reference for those entering the field.

The chapters in this new edition will highlight the progress made during the 10 years that have passed since publication of the second edition. The book is now organized into four major divisions rather than the six used in the previous

edition. Consolidation of 31 chapters into the present 25 allowed a more concise summary of most of the previous topics and inclusion of more current aspects of the disease. Although most of the previous topics have been updated (e.g., Chapters 1, 3, 6, and 16) or merged into chapters that now cover more than one subject (e.g., Chapter 15, “Clinical and Diagnostic Features in HIV Patients”), a few chapters, like the one on in vitro cultivation, have been eliminated. Rather, applications of in vitro systems have been incorporated in the appropriate chapters (e.g., Chap. 22, “Polyamines and Iron,” and Chap. 25, “In Vitro and In Vivo Testing of Compounds”). Other chapters on metabolism that focus on specific pathways and potential drug targets include chapters on lipids, purines, folic acid biosynthesis, and cytochrome B (Chaps. 21, 23, and 24). New chapter topics have been designed to represent areas of significant progress or changes in research directions. These include a description of the ongoing genome project (Chap. 7); a discussion of genetic diversity in the context of nomenclature and recognition of species (Chap. 2); and molecular typing (Chap. 19). There is an expanded section that summarizes the latest advances in the rapidly developing field of host defenses against *Pneumocystis* (Chaps. 8 to 13). Chapters are devoted to CD4 cells, CD8 cells, macrophages, cytokines, antibodies, the neonatal period, the surfactant system, and the mechanisms of lung injury. A separate chapter (Chap. 18) is devoted to immunological responses to *Pneumocystis* in humans. Other areas where exciting insights into the complex biology of *Pneumocystis* have been achieved include its life cycle, with candidates for mating types (Chap. 5), and the mechanisms of antigenic variation (Chap. 4).

Perhaps the most dramatic change in the clinical management of pneumocystosis during the past 10 years has been the routine use of highly active antiretroviral therapy (HAART) for the treatment of HIV infection, beginning in the United States in 1995. The increased immune function that occurs in most patients receiving HAART has resulted in a dramatic decline of the incidence of *Pneumocystis* pneumonia in most developed countries (Chaps. 15 and 16). However, it would be prudent not to take this decrease as a signal for complacency. Expansion of the HIV infection into persons of lower socioeconomic status, inadequate education to prevent HIV, and potential drug resistance may reverse the progress realized in the previous decade. The incidence of pneumocystosis is on the rise in developing countries, with a particularly severe impact on young children infected with HIV (Chap. 17). In the United States and elsewhere, the number of different types of drugs available for the treatment of *Pneumocystis* remains limited (Chap. 20). There is strong evidence that the target genes of the most effective therapies available for *Pneumocystis* pneumonia are acquiring mutations which, in other pathogens, provide resistance to these drugs (Chaps. 23 and 24). Since there is little apparent interest in the pharmaceutical industry in developing new anti-*Pneumocystis* agents, most of the research on this subject is being performed in academia (Chap. 25). Additionally, the development of sensitive molec-

ular techniques to detect *Pneumocystis* infection in healthy individuals and people with chronic lung diseases raises the possibility that the spectrum of illness caused by *Pneumocystis* may extend well beyond the immunocompromised host. We are now challenged with an expanding population base and a changing genetic landscape in the organism. In light of these considerations, it is then timely to reprise the current status of our knowledge regarding the basic biology, clinical status, and genetics of these resilient organisms.

Finally, we wish to acknowledge the assistance of many people who helped us put this book together. We thank the three generations of *Pneumocystis* investigators for the time and effort they devoted to their chapters. We are particularly grateful for their patience as we struggled to reach deadlines and to reach consensus on a variety of matters. One of the most contentious issues was nomenclature, and much of the Introduction to the book is devoted to this subject. We also thank other investigators who have made important contributions to *Pneumocystis* research, but because of space limitations, could not be included as authors of chapters. We wish to thank our families for their patience and tolerance of the many hours we devoted to the book. We are indebted to Sandra Beberman, Moraima Suarez, and their colleagues at Marcel Dekker not only for their cooperation and helpful advice but also for their willingness to change the title of this edition of the book from *Pneumocystis carinii* Pneumonia to *Pneumocystis* Pneumonia in recognition of what we feel are necessary changes to the field. It is our sincere hope that this edition of the book will serve all readers well.

On a sad note, we mourn the passing of our esteemed colleague and friend Ann E. Wakefield, who was the author of two chapters. She will be missed for her contributions to the field as well as her collegiality for which she will be especially remembered. This edition is dedicated to her memory.

Preface to Second Edition

Pneumocystis carinii is an organism of major medical, scientific, and public health importance. The first edition of this book, published in 1984 and edited by Dr. Lowell Young, was widely acclaimed as a valuable and authoritative reference. The dramatic changes since that time in our concepts of *P. carinii*, stemming from its association with the acquired immunodeficiency syndrome (AIDS), created the need for a second edition. To better understand these events and the revisions that have been made in the new edition, it is appropriate to briefly review the history of *P. carinii* and the conferences and publications that have been devoted to this organism.

P. carinii was discovered in the early 1900s but did not attract attention until the 1940s and 1950s, when it was found to be the etiological agent of interstitial plasma cell pneumonia. This was a disorder that afflicted premature, malnourished infants in orphanages in Europe during World War II, but has also been reported in other parts of the world where such conditions exist. In the 1960s and 1970s, *P. carinii* became recognized as a major cause of pneumonia in the immunocompromised host and thus represented a complication of medical progress. Increasing numbers of cancer patients and organ transplant recipients were being kept alive by more aggressive immunosuppressive or cytotoxic therapy; advances in immunology led to the identification and characterization of primary immune deficiency disorders. Research on *P. carinii* was inhibited by the lack of a reliable in vitro cultivation system. With the discovery of safe, effective therapy and prophylaxis for *P. carinii*, the lack of basic knowledge about the biology of the organism did not seem to be an urgent problem.

In the 1980s, *P. carinii* became recognized as the leading cause of opportunistic infection and mortality in patients with AIDS in the United States and many

countries in Western Europe. Pneumocystosis in AIDS patients was characterized by new clinical features, such as subtle presentation, extrapulmonary manifestations, and high frequency of recurrence. Major new problems for the use of standard anti-*P. carinii* drugs developed in terms of efficacy, toxicity, and cost. The tens of thousands of cases of *P. carinii* pneumonia that occurred each year created an enormous burden on the health care system, and “PCP” became well known in the media as the rallying point for activist groups demanding more government action on AIDS. These events stimulated new initiatives in basic and clinical *P. carinii* research, such as the National Cooperative Drug Discovery Groups, the AIDS Clinical Trials Units, and special grants and contracts.

The first international conference on *P. carinii* was held in December 1973, at the National Institutes of Health. Emphasis was placed on the clinical aspects of *P. carinii* pneumonia and on tracing the development of the disease from its occurrence in debilitated infants to the compromised host. The proceedings of this meeting were published as the *National Cancer Institute Monograph #43* in 1976. Fifteen years elapsed before the second conference, which was held at the University of Bristol, England, in July 1988, under the auspices of the Society of Protozoologists. This meeting focused on research and attracted physicians and basic scientists from all over the world. There were some truly startling revelations (e.g., ribosomal RNA data suggesting that *P. carinii* is a member of the fungi), which led to major changes in our concepts of the organism. Collaborative projects developed involving subjects ranging from molecular biology to drug therapy. The proceedings of this meeting were published in the January/February 1989 issue of the *Journal of Protozoology*. The third international conference, also sponsored by the Society of Protozoologists, was held at Montana State University, Bozeman, Montana, in July 1991, and the proceedings appeared in the November/December 1991 issue of the *Journal of Protozoology*. The meeting built on the success of its predecessor and, judging by the increased attendance and number of papers presented, enthusiasm for gatherings of this type will remain high for the foreseeable future.

Three books have been devoted exclusively to *P. carinii*. The first was the original edition of *Pneumocystis carinii Pneumonia*, edited by Dr. Young. This book was conceived before the AIDS epidemic became widely known; although a chapter on AIDS was included, most of the text was devoted to the pre-AIDS era. The book consisted of eight chapters written by nine contributing authors who were given considerable freedom in chapter length and content. *Pneumocystis carinii Pneumonitis*, a two-volume text written by Dr. Walter Hughes in 1987, was a systematic and thorough analysis of *P. carinii* since its discovery and included some material on AIDS. *Pneumocystis carinii*, written by Dr. Julian Hopkin in 1991, is a concise review of our current clinical and basic knowledge of the organism.

The second edition of *Pneumocystis carinii Pneumonia* has undergone major changes. Since Dr. Young was no longer actively engaged in *P. carinii* research, I was asked to become the editor. I had been a contributor to the first edition and had also served as the editor of another Marcel Dekker book, *Parasitic Infections in the Compromised Host*, published in 1989. Dr. Young's Foreword provides continuity between the two editions.

The second edition consists of 31 chapters written by more than 40 contributors. This marked increase over the first edition reflects the dramatic rise in the general level of interest in and understanding of this organism. The authors who participated in this endeavor are among the leaders in *P. carinii* clinical care and research. The book has been designed to present a balanced and comprehensive examination of the latest advances in this field, and to contain the most up-to-date references. There has also been an attempt to include topics (e.g., drug development) that have received little formal attention in the past.

Chapters in the book have been arranged according to the traditional subject headings. A major effort has been made to allow the authors latitude to express their opinions while at the same time giving careful attention to issues of clarity, continuity, and avoidance of duplication. However, as with any multiauthored text, some areas of controversy and overlap were unavoidable.

Although this book is intended as a reference text on *P. carinii*, I believe it will have wide appeal to clinicians and basic scientists who are interested in AIDS and opportunistic infections.

Peter D. Walzer

Introduction

The history of organisms collectively known as *Pneumocystis* has been plagued with misunderstanding, disagreement, and controversy. The first description of these organisms by Carlos Chagas resulted in a case of mistaken identity. Featured as a part of the life cycle of trypanosomes in 1909 [1], these microbes were defined as a single genus and species, *Pneumocystis carinii*, 3 years later [2]. Soon forgotten, *P. carinii* fell into obscurity until three decades later, when European investigators identified it as the causative agent of epidemics of plasmacellular interstitial pneumonia (also known as “interstitial plasma cell pneumonia”) that were rampant in European orphanages, where premature, malnourished infants and small children resided as a result of World War II. American pediatricians disagreed with their colleagues overseas and were slow to accept the role of this protozoan parasite as the primary cause of the pediatric pneumonia. They were more convinced that a viral agent was the culprit [3]. Cases of plasmacellular pneumonia continued to be reported in Europe, the United States, and elsewhere during the next two decades. These were almost always associated with underlying conditions that caused debilitation of the immune system and with the presence of the small protozoan parasite *P. carinii*.

With the advent of aggressive chemotherapy and cytotoxic agents for cancer therapy, cases of *Pneumocystis* pneumonia surged in the 1960s and 1970s [4]. Clinical management of the infection was significantly improved with identification of the combination of trimethoprim-sulfamethoxazole as an effective therapy [5]. Already hindered by a lack of an in vitro cultivation system and difficult animal models, research on these enigmatic organisms languished. This situation changed dramatically in the 1980s, when an immediate and heightened interest resulted from recognition of *P. carinii* and the pneumonia it caused (PcP) as a

primary source of mortality among AIDS patients [6–8]. Clinical, basic, and applied research efforts were mobilized, and an explosion of information regarding the nature of the organism and the disease it caused ensued.

Publication of the previous edition of this book in 1994 brought the medical and research community up-to-date on the state of the basic biology, epidemiology, pathophysiology, clinical features, diagnosis and treatment as well as prevention. At that time, the fungal nature of the organism revealed by the relatively new science of molecular phylogenetics drew the most controversial response from opponents who argued its protozoan affinities. Coincident with the growing collection of gene sequences and cross-inoculation experiments came the realization that *Pneumocystis* populations from different mammalian hosts were very distinct entities. It was clear to the majority of participants that continuing to use “*Pneumocystis carinii*” as the name of the organism from any mammalian host was leading to confusion in the literature and in GenBank, the database for all gene sequences. So, the organisms that once gathered under the rubric “*Pneumocystis carinii*” were separated into “special forms” based on their choice of mammalian host (e.g. *Pneumocystis carinii* f. sp. *hominis* was the organism infecting humans) [9]. As additional sequence comparisons and phenotypic (antigenic) differences provided further evidence for these separations, the scientific community again met and decided to begin the formal process of naming species in the genus *Pneumocystis* in 2001 [10]. A description of the human organism, named *Pneumocystis jirovecii* [11], has contributed to much of the present-day controversy that continues to swirl about these small microbes [12–14]. Nonetheless, efforts to formally name species within the *Pneumocystis* genus are proceeding.

The decision to begin formal description and the naming of different species of *Pneumocystis* was not done precipitously but with much deliberation and genuine efforts to involve the entire scientific community concerned with *Pneumocystis*. These efforts are evidenced by published summaries of the roundtable discussions that occurred in 1994 [9] and 2001 [10], resulting from the international workshops focusing on *Pneumocystis* research. The summary in 2001 includes a guide to the requirements of International Code of Botanical Nomenclature (ICBN) for those investigators wishing to describe and name a new species, which can be a daunting task. Description of *Pneumocystis* species must follow the guidelines set out in the (ICBN), since *Pneumocystis* is recognized as a member of the fungal kingdom [15].

As mentioned above, a provisional nomenclature to differentiate the *Pneumocystis* populations found in mammalian hosts was adopted in 1994, following the ICBN (article 4, note 3) for “parasitic forms, especially fungi, characterized by their adaptation to different hosts, but scarcely or not at all from a morphological standpoint.” The host of origin was used to delineate the population in this tripartite nomenclature. This naming convention should still be used to differentiate those organisms that have not yet been formally described. At the time this

chapter went to press, there were four recognized species of *Pneumocystis*: *P. jirovecii* Frenkel 1999; *P. carinii* (Delanoë and Delanoë 1912) Frenkel 1999 [11]; *P. wakefieldiae* Cushion et al. [16]; and *P. murina* Keely et al. [17]. Frenkel first named the *Pneumocystis* species found in human beings as *P. jirovecii*, to honor Otto Jirovec [18], an early worker in the *Pneumocystis* field. Dr. Frenkel re-described *P. jirovecii* according to the ICBN in 1999 [11]. In the same article, he described a rat *Pneumocystis*—*P. carinii*, according to the ICBN—thus invalidating the original description by Delanoë and Delanoë 1912. It should be noted that the correct species name should be “*jirovecii*” rather than “*jiroveci*,” in accordance with recommendations 23A and 60C of the ICBN. This correction does not affect its valid date of publication (Article 45.2). In 2004, two additional species were described in rodent models. *P. wakefieldiae* is the second species that infects rat lungs (previously called “variant and *P. carinii* f.sp. *ratti*”) and was named to honor Dr. Ann E. Wakefield, a departed colleague (see dedication). The species infecting mice (there appears to be but one) was named *P. murina*, based on the host of origin.

Naming of the different *Pneumocystis* populations has not been embraced in part or in whole by all members of the scientific community and remains controversial. Recognizing that the field is in a state of flux regarding this matter and respecting the opinions of our authors, we, the editors, nonetheless strove to standardize the nomenclature used in these chapters, but gave our authors the final choice. Thus you will see a variety of naming conventions used throughout the book, reflecting this situation. We asked the authors to follow the ICBN regarding the recognized *Pneumocystis* species and special form names. We further suggested that “*Pneumocystis*” be used in referring to the genus in general or collectively. In some cases, the species referred to could not be identified, as in the rat model, which is infected by two species, *P. carinii* and *P. wakefieldiae*; these organisms were referred to as simply *Pneumocystis* from rat or rat *Pneumocystis*. PcP could still be used to refer to the disease state in any host, since the abbreviation was redefined as *Pneumocystis pneumonia*. “Pneumocystosis” could also be used to refer to PcP or *Pneumocystis* infection.

When using gene names, we recommend the following outlined suggestions by Stringer and Cusion [19]:

1. Genes will each be symbolized by three italic letters. In cases where function is known or is suggested by DNA or peptide sequence homology, the three-letter italic symbol will be derived from function. Genes that function in a related way will have the same three-letter symbol followed by an italic numeral. For example, *mpk1*, *mpk2*.
2. Alleles of a gene: the first allele (i.e., sequence) that is found will be designated with superscript 1, such as *xyz*¹, and any subsequent alleles will be designated as *xyz*², and so on.

3. Gene families: genes that are members of gene families will all be given the same three-letter italic name followed by an italic numeral. Each gene in the family will have a different numeral appended, such as *msg1*, *msg2*, etc. The entire gene family will be symbolized by the same three letters written in capital roman type, such as MSG.
4. Proteins: when possible, a protein will be symbolized by the same three letters as the gene which produced that protein, but the three letters will be written in roman type, with the first letter an uppercase, such as Xyz1, Msg1, etc.

Finally, we asked our authors to use fungal terms in referring to the life cycle/developmental stages of *Pneumocystis*; *trophic form* instead of *trophozoite*; *sporocyte* for precyst; *spores* for intracystic bodies; and *spore case* for cyst. The process of encystment and resultant spore production is sporogenesis. The process of excystation is spore release. Again, the personal preference of each author was respected and the variation that the reader will observe throughout the book shows that the controversy surrounding *Pneumocystis* is alive and well.

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1

Historical Overview

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I. Introduction

The history of *Pneumocystis carinii* opens with a series of seemingly trivial events beginning around the turn of the twentieth century [1]. Its emergence from an obscure, insignificant infection of rodents to a major, life-threatening infectious disease of humans over only a few decades was due to changes in the human host, not the organism. Remarkable in modern medicine is the fact that the discovery of *P. carinii* was accomplished in great part by simple studies of human and animal lung tissues with the compound microscope —as were the elucidation of its structure, pathogenesis, epidemiology, and host response mechanisms and also the development of therapeutic and prophylactic agents against it. Only in the last decade and a half have the powerful tools of molecular biology been applied to *P. carinii*.

II. 1908 to 1913

The story of *P. carinii* began in Brazil in 1908. At that time the capital, Rio de Janeiro, was one of the most beautiful cities in the world. Among the many magnificent civic buildings of government, commerce, performing arts, and religion, one stood truly unique among others of North and South America: the

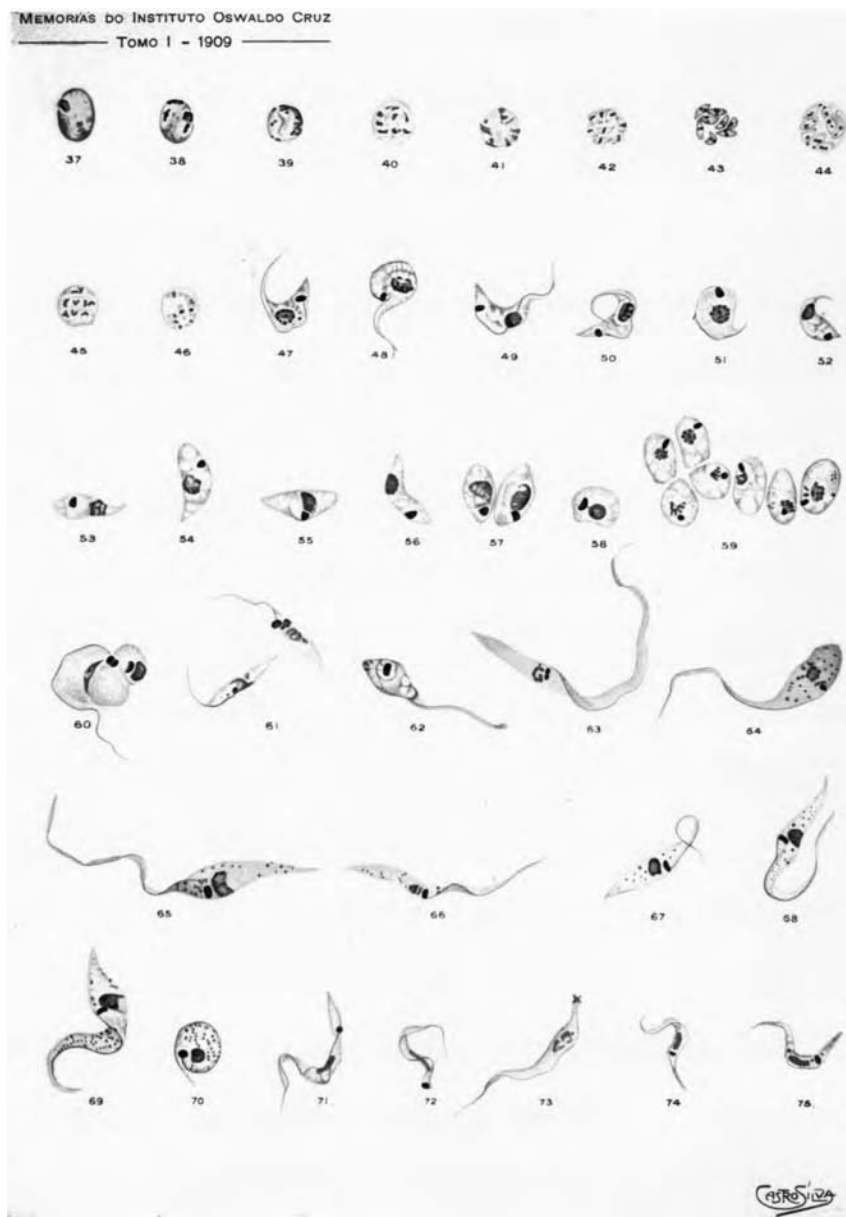


Figure 1 A. Color plate from Chagas' study in 1909 showing stages 37 through 75 of the 109 stages in his proposed life cycle of *Trypanosoma cruzi* (*Schizotrypanum cruzi*). Stages 37 through 46 are believed to be *Pneumocystis carinii* [2]. (continues)

Institute of Experimental Pathology, which was opened in 1908 for research in the field of infectious diseases. Its Moorish architecture—with domed towers projecting skyward from each corner—looked as much like a cathedral as a scientific laboratory. The laboratories were spacious and well equipped, a compelling factor for the recruitment of promising young scientists from Europe. The institution was first called the Manguinhos Institute, but the name was soon changed to the Oswald Cruz Institute to honor the great Brazilian physician whose efforts led to the eradication of yellow fever from Rio de Janeiro. It was here that Carlos Chagas, a 29-year-old protege of Cruz, found a new parasite that he called *Trypanosoma cruzi*, the cause of a life-threatening disease of workers building a railroad in the hinterland of Brazil. This discovery alone gave Chagas a prominent and permanent place in the history of medicine.

Chagas was able reproduce the disease by injecting the parasite into rats and guinea pigs. He identified various forms of *T. cruzi*. In histological preparations of the lungs, he began to visualize forms never before seen in the new disease known as trypanosomiasis. These were round to cup-shaped, cyst-like structures, a little smaller than red blood cells. Some of these “cysts” contained daughter cells, up to eight in number. He concluded that these forms were stages in the life cycle of *T. cruzi*.

Chagas recalled lung specimens from a human case he had studied earlier, while investigating this disease along the railroad line. A malnourished 4-year-old child died, and the autopsy showed the lungs to be filled with the same frothy fluid and peculiar form of the parasite he saw in the laboratory animals. In a 60-page article published in 1909, Chagas described the complete life cycle of *T. cruzi* based on the finding of the new cyst-like forms. So important was his finding that the article included drawings of the 109 stages of the life cycle printed in color, a rare occurrence in that day (Fig. 1) [2].

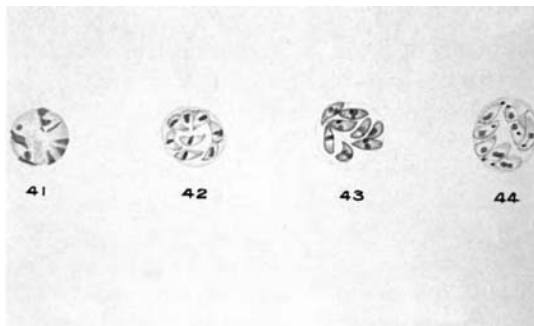


Figure 1 (continued) B. The cyst forms of *P. carinii* stained with Giemsa stain, demonstrating eight “sporozoites” and a poorly staining cyst wall (2).

Now the scene changes from Rio de Janeiro to São Paulo, also a thriving Brazilian metropolis, some 200 m away. There lived Antonio Carini, a contemporary of Chagas and a well-trained scientist (Fig. 2). Born in Sondrio, Italy, Carini was 7 years older than Chagas. He had studied medicine in Pavia and, after graduation, took a doctorate in microbiology at the University of Berne. In Switzerland, he became chief of the Vaccine and Serotherapeutic Institute. In the early 1900s, however, he moved to São Paulo to become director of the new Pasteur Institute of São Paulo and professor of microbiology at the University of São Paulo [3].

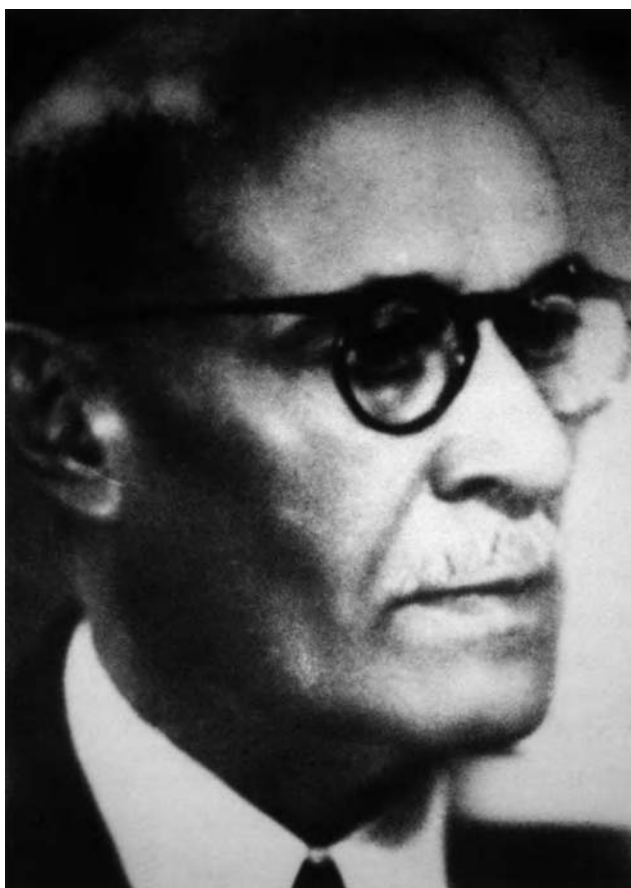


Figure 2 Professor Antonio Carini, 1872–1950 (Courtesy of Professor Carlos da Silva Lacaz, University of São Paulo, Brazil.)

Once in São Paulo, Carini began to search for Chagas' new cellular forms. He infected rats with *Trypanosoma* species, removed the lungs in the same manner as Chagas had done, mounted slices of the tissue on glass slides, and observed the same cystic forms described by Chagas. Carini reported his findings at a meeting of the Medicine and Surgery Society of São Paulo, and in 1910 his research was published in the *Bulletin of the Institute Pasteur* [4].

Carini was unsure about the cysts as a part of the life cycle of trypanosomes. In some animals, they were absent even when trypanosomiasis was extensive. Carini sent his data and specimens to Felix Mesnil, a friend at the Pasteur Institute in Paris. In his laboratory, simple and careful studies by Pierre and Mme. Delanoë showed that the organism discovered by Chagas and Carini could be found in the lungs of Parisian rats without trypanosomes, proving it to be unassociated with the *Trypanosoma* species. They named this new organism *Pneumocystis carinii*, because cysts were found only in the lungs and Carini had provided the specimens for study.

Because of the importance of this classic three-page article published in French and not readily available in English, some excerpts from “*Sur les rapports les kystes des Carini des poumon des rats avec de Trypanosoma lewisi*” by the Delanoës in 1912 [5] are translated here. After a detailed description of the morphology of the organism precisely as we know it today, when stained with Giemsa, the results were described as follows:

Through a series of rather fortunate circumstances, our latest research on new adult rats [uninfected with trypanosomes] has met with success: in 22 rats we found 22 with Carini cysts. Five of these rats came from outside sources. Upon a direct blood test, they were identified as being clear of *T. lewisi*. Their lungs were unevenly and slightly parasitized. Seventeen other rats came from the farm of Mr. Borrel at Pasteur Institute. Their lungs had many cysts. These 17 rats were seen to be free of *T. lewisi* upon repeated direct blood tests. Furthermore, among eight of these, we took blood from the heart with which we then inoculated an equal number of young rats that did not become infected, though they were sensitive [susceptible] to *T. lewisi*.

Carini cysts are susceptible of transmission by direct contagion. Two females from the Borrel farm were carefully cleared of mange [scabies] by means with several treatments with Helmerich salve. They were then placed in new jars, where they gave birth. This group was completely protected from all ectoparasite contamination. Three months later the four little ones and the two mothers were seen to be carriers of cysts.

Doubtless, Carini pneumocysts represent a new parasite in rats. Should they constitute a new kind, we propose to give it the name of *Pneumocystis carinii*. Affinities should be investigated, perhaps along the lines of *Coccidia*.

Is it not remarkable that these investigators, supplied with less than 35 rats and a microscope, established *P. carinii* as a new entity, noted that the infection occurs only in the lungs, and—by placing two pregnant rats in a jar—showed a

contagious mode of transmission to mothers and infants? One must conclude that, by rights, the name of the organism should be *Pneumocystis delanoe*, but these humble investigators suggested the name of *Pneumocystis carinii* in honor of the second person to describe it and who interpreted his findings erroneously. (A complete translation of the article may be found in Ref. 6). The Delanoes described in their second and final paper their finding of *P. carinii* cysts in one of ten guinea pigs and no cysts in one rabbit, one frog, and three eels [7].

Following the publication of the Delanoes' paper in 1912, both Chagas and Carini acknowledged publicly that they had made mistakes and that, indeed, the Delanoes were correct in their conclusions that the microbial forms they thought were trypanosomes were proven to be a new organism, *P. carinii*. The name seemed acceptable to everyone because the organism was merely an insignificant contaminant. From that time on not one of the key players—Chagas, Carini, or the Delanoes—pursued further research on this organism and never again wrote of it in published literature. This tricky microbe had embarrassed Chagas profoundly. Nevertheless, his many other contributions clearly outweigh this rather insignificant error. Carini, though not blameless, had aided in seeking the truth by cooperating with and supplementing the work of the Delanoes. He went on to a long and meritorious career in Brazilian medicine at the Pasteur Institute in São Paulo [3]. After some 40 years in Brazil, Carini returned to Italy, his homeland, and died there in 1950 at the age of 78. None of these investigators came to appreciate that their mischievous little cysts were anything more than innocuous and unimportant parasites of rodents. Neither they nor other scientists had enough curiosity to search out the nature of *P. carinii*. Does it cause some disease? Is it contagious? Do other animals harbor it? Could it affect humans in any way? How prevalent is it if readily found at such distant sites and climes as Brazil and France?

III. 1913 to 1917

From 1913 to 1917, *P. carinii* was described in rats, guinea pigs, monkeys, and rabbits in Brazil [8], rats [9] and mice [10] in England, and rats in Switzerland [11].

IV. 1917 to 1942

For almost three decades after the discovery of *P. carinii*, nothing further happened. Not a single scientific publication referred to this organism. It became lost in obscurity.

V. 1942 to 1960

A. Interstitial Plasma Cell Pneumonitis in European Infants (1942 to 1960)

Around the time of World War II and later, a mysterious new type of pneumonitis was appearing in Europe [12]. It targeted the lungs of infants and was limited

precisely to those from about 2 to 8 months of age. Furthermore, it was in great part limited to infants born prematurely or those who were normal at birth but later became debilitated because of poor nutrition. Nursing wards for infants were especially vulnerable. The onset of illness was subtle, starting with a decrease in feeding, restlessness, poor weight gain, and diarrhea at times. Then these infants' breathing was noted to be more rapid than usual, even though there was no fever. Daily the condition worsened and breathing became labored, with respirations up to 100 per minute. Some babies had weak coughing and others were unable to cough and cry because they could not slow their breathing long enough to utter any sound beyond a weak grunting noise. Along with intercostal retractions, neck muscles stood taut, and the nasal alae flared with each breath as if the child were gasping for more air. The abdomen became large and distended. Most striking was the blue-gray color of the skin, accentuated by extensive blueness of the lips and whites of the eyes. Their frightened faces suggested the infants' awareness of their plight. The condition progressively worsened over 1 to 2 weeks; some died of exhaustion, while others struggled with the disease for a month or so and eventually recovered. No drugs affected the disease; only the oxygen tent provided a somewhat tranquilizing effect for some babies, although this was often only transient.

The first cases of interstitial plasma cell pneumonitis were described in Berlin in 1938 [13], but reports of the syndrome of this pneumonia of unknown etiology in infants soon began to appear throughout central, northern, and western Europe. Over 700 cases were reported from Switzerland between 1941 and 1949 [14], and the pneumonitis was commonly found in Czechoslovakia, Italy, Hungary, Germany, Yugoslavia, Austria, Denmark, Sweden, France, and Finland in the early 1950s [12]. One case was reported from England in 1955 [15]. Medical investigators could find no explanation for the malady. There was some hint of contagion, in that infants in certain nurseries were more likely than those in others to have the disease. Searches for viruses, bacteria, and fungi as causative agents were unrevealing. In none of the cases were other members of the family affected. Some suspected a yeast and others favored a new microbial cause [12]. The injection of samples of diseased human tissues into experimental animals did not reproduce the disease.

For more than a decade, mothers of newborn infants feared that this pneumonitis would affect their babies. Doctors and nurses seemed helpless at the cribsides of the suffocating infants.

B. *Pneumocystis carinii* Associated with Interstitial Plasma Cell Pneumonitis of Infancy (1942 to 1960)

In 1942, van der Meer and Brug [16], two physicians in the Netherlands, reported three infants with pneumonitis studied at autopsy; in these cases, they clearly

implicated *P. carinii* as the cause of the infection (Fig. 3). However, their study was reported in a Dutch medical journal during World War II, and it went unnoticed for many years.

A breakthrough came in 1951, when Vanek in Czechoslovakia discovered *P. carinii* in the lungs of 16 infants who had died of the mysterious pneumonitis [17]. The youngest of these was 4 months of age, 10 were premature, and 6 were described as dystrophic, meaning debilitated. Careful microscopic examination by Vanek and his coworkers revealed hundreds of thousands of cysts and smaller forms of *P. carinii* filling the alveoli. No wonder these poor infants gasped for air and turned blue; they were drowning in a sea of organisms. Thus Vanek acknowledged the discovery that van der Meer and Brug had made a decade earlier [18].

Subsequent studies by Vanek and Jirovec and Lukes, his close coworkers, were published in both Czech and German medical journals [18]. Their work was accepted by physicians as explaining the cause of the epidemic of interstitial plasma cell pneumonitis that was plaguing European nurseries. Soon other reports—from Germany, France, Yugoslavia, England, Austria, Italy, and

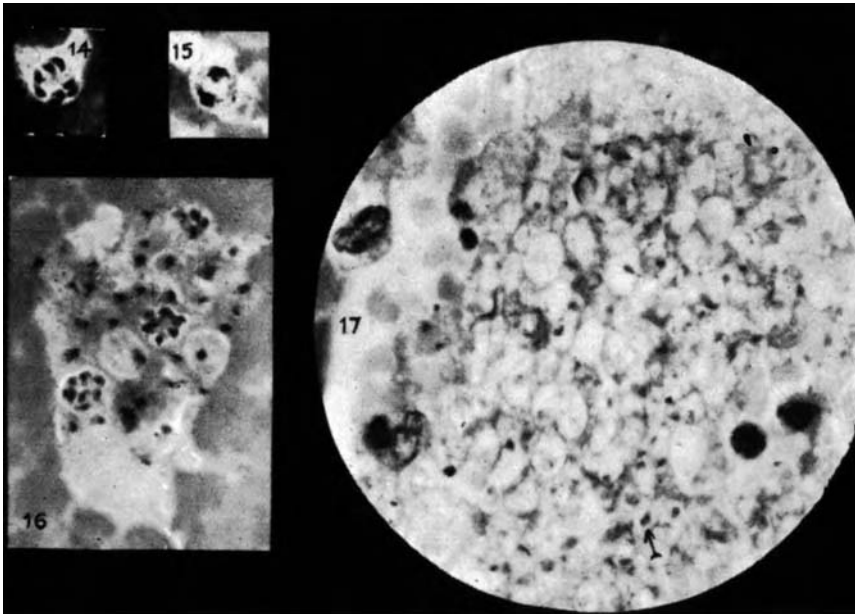


Figure 3 Photomicrographs from the article by van der Meer and Brug in 1942 showing typical *P. carinii* “cysts” and “trophozoites” (14, 15, and 16 subfigures) and the typical proteinaceous “honeycomb” exudate in the alveolus [16].

Hungary—described similar findings. For any serious scholar of *P. carinii*, a monumental article by Gajdusek published in *Pediatrics* in 1957 is required reading. Here he chronicles the history of *P. carinii* with vivid accounts of the European cases. This article introduced American physicians to the entity [12].

C. *Pneumocystis carinii* in Adults (1942 to 1960)

Prior to 1956, *P. carinii* infection was considered a disease limited to early infancy. In 1956, Hamperl drew attention to the entity with his report of three cases in adults [19,20]. From 1956 to 1960, a total of 15 additional cases of *P. carinii* pneumonitis were found in adults [21,22]. Woodward and Sheldon [23] found subclinical *P. carinii* in two adults: a 49-year-old man with acute lymphocytic leukemia and a 52-year-old man with lymphoma.

It is noteworthy that the population of immunocompromised adults in the 1950s was remarkably small. Immunosuppressive anticancer therapy was in its early stages, the use of cortisone was limited, many cancer patients died early in the course, and organ transplantation was not established.

D. *Pneumocystis carinii* in the Western Hemisphere (1942 to 1960)

In July 1956, Dauzier, a resident in Pathology at Norwalk Hospital, and colleagues [24,25] in Connecticut reported the first documented case of *P. carinii* pneumonitis in the United States. Four months later, Russell and Nelson [26] published descriptions of two infants with the pneumonitis in Oklahoma City. A few cases of interstitial plasma cell pneumonitis had been described earlier, but the organism was not identified [27]. Also in 1956, Gagne and Hould [28] described three cases of *P. carinii* pneumonitis in Canada, which had occurred in 1953 and 1954. Subsequently, Berdnikoff et al. [29] found, by retrospective study of autopsy specimens, 14 cases of *P. carinii* pneumonitis in Canadian infants who had died from 1930 to 1958. The first two cases in Canada were infants who died in 1930.

Ariztia et al. [30] reported 10 patients who died of *P. carinii* pneumonitis at Luis Calvo Mackenna Children's Hospital in Santiago, Chile. These cases were reported in 1957 and represent the first documented cases in South America. Ironically, almost 50 years earlier, Chagas, in Brazil, had made the same association of this organism in the lungs of a human but mistakenly called it a cyst form of a trypanosome.

In 1959, Sheldon, a pathologist at Emory University in Atlanta, made some important contributions to our understanding of *P. carinii* pneumonitis [31,32]. Initially, he discovered *P. carinii* to be the cause of fatal pneumonitis in two patients. One case was a seemingly normal 3-month-old infant who was found dead in her crib. The second case was not an infant but rather a 10-year-old girl with glomerulonephritis who died in renal failure after a course of corticosteroid

therapy. These cases were the first to suggest that *P. carinii* infection might be associated with sudden infant death syndrome (SIDS), to be elucidated by Vargas et al. [33] more than 40 years later, and that the pneumonitis might occur in older individuals following treatment with cortisone, a relatively new drug at the time. It is worth noting that investigations of *P. carinii* infection up to that time had focused almost entirely on infants with interstitial plasma cell pneumonitis, and no serious search had been made in older children and adults. The fact that the organism does not stain with the hematoxylin and eosin stain used for routine histopathology hindered its detection in routine autopsy studies.

Sheldon became interested in *P. carinii* with the study of his first patient and devised experiments in an attempt to reproduce the disease in experimental animals. His observations were important and added to the foundation of knowledge leading to the use of animal models in the study of this disease.

E. Discovery of the Animal Model for *Pneumocystis carinii* in the Immunocompromised Host (1942 to 1960)

In 1955–1956, Weller [34,35] in Germany attempted to infect rats with *P. carinii* by dipping the noses of the animals in a suspension of lung tissue from a child dying of *P. carinii* pneumonitis. He also administered cortisone for immunosuppression and penicillin to prevent death from bacterial infections. After several weeks of this treatment, the inoculated animals had pneumonitis with *P. carinii*; however, pneumonitis was also found in the uninoculated animals. Weller concluded that immunosuppression activated latent infection, even though he did not search for the organism in the untreated rats.

Sheldon's experiments [31] involved three groups of young rabbits: one group was inoculated with *P. carinii* and given cortisone and penicillin; the second group received saline or boiled infected lung tissue with and without the immunosuppressive therapy; and the third group served as untreated normal controls. The results showed extensive *P. carinii* pneumonitis in both the inoculated and uninoculated cortisone-treated groups but not in the normal controls, confirming the earlier findings of Weller that the organism can readily be acquired or activated from a latent state in the immunocompromised host.

The cortisone-treated animal model would later serve as a major system for research relevant to the human infection, because the disease in the animal model was remarkably similar to that in humans.

F. Pentamidine: First Effective Drug for *P. carinii* Pneumonitis (1942 to 1960)

The first drug shown to be effective against *P. carinii* pneumonitis was pentamidine isethionate. In 1958, Ivady and Paldy [36] described three patients who recovered from *P. carinii* pneumonitis following the intramuscular administration

of this drug. More convincing was their study reported in 1963, where the mortality rate of 50% among untreated infants fell to 20% when pentamidine therapy was used [37]. The rationale for a trial of pentamidine for *P. carinii* pneumonitis was that the drug had been found effective in the treatment of trypanosomiasis, and, *P. carinii* “looked something like” a trypanosome. Pentamidine was the only drug known to be effective for *P. carinii* pneumonitis and was the standard of therapy until the mid-1970s.

Eventually the epidemics and endemics of *P. carinii* pneumonitis in European infants subsided along with the economic recovery following World War II and improvements in living conditions.

VI. 1960 to 1980

The next era for *P. carinii* emerged in the 1960s, not in debilitated European infants but in children and adults who had cancer or who had been born with defective immune systems. These cases occurred in major medical centers in the United States and only in those patients whose immune systems had been impaired by chemotherapy, radiation, or birth defect. Then as organ transplantation came into use, the necessary suppression of the immune system became associated with the complication of *P. carinii* pneumonitis.

A. Cancer (1960 to 1980)

Because *P. carinii* was not a reportable disease to the U.S. Public Health Service and no single institution had a sizable population of cases, few data were available on the epidemiology of this infection in the United States until the important study of Walzer et al. in 1974 [38]. This study took advantage of the fact that pentamidine was the only drug available for the treatment of *P. carinii* pneumonitis and the only source of the drug in the United States was through the Parasitic Disease Drug Service at the Centres for Disease Control (CDC). As a part of the request for the drug, physicians were required to provide clinical data about their patients. This database allowed some important observations. From November 1967 to December 1970, requests for pentamidine were received for 579 patients. The diagnosis was confirmed histologically in 194 patients. The underlying diseases were leukemia, 91 cases (46.9%), Hodgkin's disease and lymphomas, 34 cases (17.5%); solid tumors, 7 cases (3.6%); primary immunodeficiency disorders, 25 cases (13.0%); organ transplant recipients, 22 cases (11.3%); collagen-vascular disorders, 9 cases (4.6%); and, miscellaneous/unknown causes, 6 cases (3.1%). This study provided a glimpse of the magnitude of the disease at the time, although denominator data on the populations of immunocompromised hosts at risk were not available. About the time of this report, new drugs were evolving, so that physicians could obtain optimal therapy from local pharmacies and hospitals

without CDC resources. Thus a useful system for collecting data on this still somewhat uncommon infection was thwarted.

Prior to the CDC analysis in 1974, *P. carinii* pneumonitis was causing problems at several cancer centers [38]. By 1960, immunosuppressive chemotherapy for cancer was coming into general use. As the intensity of anticancer treatment increased, the prevalence of *P. carinii* pneumonitis increased [39]. In 1962, Hendry [40] reported 13 cases of the pneumonitis from Duke University in North Carolina. Nine of these patients had lymphoproliferative malignancies. In 1963, Kossel [41] described 13 children with interstitial plasma cell pneumonitis who had been given prolonged corticosteroid therapy. In 1965 Esterly [42] found 12 patients with *P. carinii* pneumonitis who had lymphoproliferative malignancies. From 1965 to 1970, 15 cases of *P. carinii* pneumonitis were documented at the M. D. Anderson Cancer Center in Houston [43]. Luna, in 1967, found the pneumonitis in a patient with neuroblastoma [44].

The problem became more prominent, both in adult and childhood cancer patients, during the 1970s. Outbreaks of *P. carinii* pneumonitis were reported from cancer centers. The number of such cases had reached the point at St. Jude Children's Research Hospital in Memphis, a large pediatric cancer hospital, that the CDC was led to investigate the epidemic, concluding that the infection was related to the intensity of anticancer therapy [45,46]. Paradoxically, this intensive chemotherapy was curative for many patients with acute lymphocytic leukemia. Overall, between 1962 and 1972, 51 cases of *P. carinii* pneumonitis occurred in a group of 1251 children with malignancies [39,47]. Elsewhere, the Children's Cancer Study Group found 22 cases of the pneumonitis among 844 children with acute lymphocytic leukemia [48] from June 1972 to February 1975. A cluster of 11 cases occurred within a 3-month period at Memorial Sloan Kettering Cancer Hospital in New York [49]. Corticosteroid therapy had been stopped in 7 patients with leukemia or lymphoma shortly before the diagnosis of *P. carinii* pneumonitis. In other studies, the occurrence of pneumonitis due to this organism was related to the intensity of chemotherapy [39]; it was noted to occur in remission as well as relapse states [50]; found to recur after successful treatment [51]; and the clinical manifestations were related to the number of cysts in lung parenchyma [52].

By 1973, the problem of *P. carinii* pneumonitis had reached the point that an international symposium on *P. carinii* pneumonitis was convened at the National Institutes of Health by Robbins, DeVita, and Dutz. This was the first major symposium to focus on this infection, and the important events were published in monograph form in 1976 [53]. Dissemination of information and discussions at this meeting spurred widespread awareness and interest in this unusual infection.

B. Primary Immunodeficiency Disorders (1960 to 1980)

It may at first appear surprising that the earliest cases of *P. carinii* pneumonitis associated with primary immunodeficiencies were found in the 1950s in infants

and children with agammaglobulinemia or hypogammaglobulinemia [54–56]. This may be partly due to the more limited understanding and capability to diagnose T-lymphocyte abnormalities at that time. We now know that this pneumonitis occurs predominantly in patients with impaired cell-mediated immunity rather than gamma globulin deficiencies.

By 1973, some 80 cases of *P. carinii* pneumonitis associated with primary immunodeficiency disorders had been reported. In that year, two excellent studies were published: one describing in great detail 15 children with the pneumonitis and congenital immunodeficiency syndromes from the University of Minnesota [57] and the second from the CDC in Atlanta, reviewing 25 cases of *P. carinii* pneumonitis in children with primary immunodeficiency disorders [58]. In both reports, severe combined immunodeficiency was the most commonly encountered type of immune deficiency disease, occurring in 67% of the Minnesota cases and 60% of the CDC cases. Subsequently *P. carinii* pneumonitis was reported in essentially all types of primary (congenital) immunodeficiency disorders. It is not uncommon for the pneumonitis to be the presenting illness in severe combined immunodeficiency syndrome (SCIDS), serving as a diagnostic indicator, much like progressive vaccinia from smallpox vaccination pointed out such disorders in infants before 1970.

C. Organ Transplantation (1960 to 1980)

The intensive efforts to ablate the immune system for organ transplantation was associated with complications of *P. carinii* pneumonitis early in the era of transplantation. In 1967, Hill et al. [59] found the pneumonitis in 9 (15%) of 60 patients who had received liver, renal, or spleen grafts. The pneumonitis was also found in 1 (5%) of 20 cardiac transplants [60]. Lung transplant recipients were highly susceptible to this infection [61]. In 1971, Solberg et al. [62] found *P. carinii* pneumonitis to be one of the infections complicating bone marrow transplant recipients. Between 1969 and 1977, *P. carinii* pneumonitis occurred in 28 (10%) of 275 patients in Seattle who had undergone allogeneic bone marrow transplantation [63].

D. Malnutrition (1960 to 1980)

From the descriptions of European infants with *P. carinii* pneumonitis, one is led to suspect that undernutrition might have been a provocative factor for immunodeficiency [64]. However, data on the nutritional status of these infants is lacking. In 1974, Hughes et al. investigated the role of malnutrition on the provocation of *P. carinii* pneumonitis [65]. A case-matched control study of 44 leukemic children with *P. carinii* pneumonitis and 44 leukemic children with pneumonitis not due to *P. carinii* showed those with *P. carinii* had significantly lower body weights and serum albumin levels. More suggestive data came from the

study of lungs of 39 children who died of kwashiorkor in South Africa compared to 21 well-nourished controls. Three (7.7%) of the malnourished patients had *P. carinii* in the lungs, compared to none in the well-nourished controls. Conclusive studies in animals showed that *P. carinii* pneumonitis could be provoked solely by dietary deprivation of protein and that intervention with protein supplements was associated with clearing of the infection [65].

E. *Pneumocystis carinii* in Normal Individuals (1960 to 1980)

P. carinii has been found sporadically in the lungs of presumably healthy humans and lower animals without evidence of disease [66]. In an autopsy study of lungs from 200 patients dying at a large university hospital in Maryland, one immuno-competent patient (hip fracture) had the organism in the lungs [67].

Anderson and Barrie reported a fatal case of *P. carinii* pneumonitis in an adult in 1960 in Toronto [68]. No underlying disease was found in this otherwise normal 36-year-old Japanese man. An attempt was made by Robinson in 1961 to determine the incidence of *P. carinii* infection by postmortem examinations in adults [69]. Of the 203 autopsies performed at the Veterans Administration Hospital in Lake City, Florida, he found 2 cases with *P. carinii* infection in the lungs. One patient was a 37-year-old man with Hodgkin's disease and the other was a 38-year-old longshoreman with chronic peritonitis and emaciation.

Serological studies of normal populations in 1977 in The Netherlands and in 1978 in the United States provided strong evidence for the high frequency of *P. carinii* infection in the normal human host. Meuwissen et al. studied antibodies in the sera from 313 healthy blood donors, 281 asymptomatic children, and 117 children with leukemia [70]. The results suggested that most people become infected with this organism, that these infections begin to occur by 6 months of age, and that by age 2 years, essentially all children have detectable antibody titers. The second study, by Pifer et al. in the United States, showed that 83% of children have antibody to *P. carinii* by 3 to 4 years of age [71]. Herrod et al. studied the in vitro response of human lymphocytes to *P. carinii* antigen and found positive responses to the antigen in 14 (88%) of the 16 adults and only 2 (13%) of the 16 cord blood specimens [72].

A distinction must be kept in mind between *P. carinii* pneumonitis and the finding of *P. carinii* in lung tissue without evidence of host response. Microscopic histological slide specimens are remarkably small samples relative to the massive tissue of the human lung. Nevertheless, clinical illness is correlated with the extent of *P. carinii* infection in histological sections [52]. Serological methods to detect antibody to *P. carinii* were insufficiently sensitive and specific to be of value in the diagnosis of individual cases of *P. carinii* infection.

F. Treatment and Prevention (1960 to 1980)

The development of all the drugs currently in use for the treatment and prevention of *P. carinii* pneumonitis came from trial-and-error approaches in humans and

animals with the infection. The early studies of pentamidine reported by Ivady and Paldy in 1958 [36,37], mentioned earlier, were continued over the ensuing 5 years, so by 1963, a total of 212 infants with *P. carinii* pneumonitis had been treated with the drug [73]. During the first 2 years of the study (1958–1959), the mortality decreased from the earlier 50% to about 20%; in the next 2 years (1960–1961), the mortality rate was further reduced to around 3%. Despite the lack of a randomized controlled study, physicians came to accept pentamidine as an effective drug for the treatment of *P. carinii* pneumonitis. However, because it required parenteral administration, was ineffective in the prevention of *P. carinii* infection in rats [74,75], and had adverse effects, pentamidine could not be used for prophylaxis.

Pentamidine isethionate was manufactured by May and Baker, Ltd., in Dagenham, England, under the trade name of Lomidine. When cases of *P. carinii* pneumonitis began to occur in the United States, the drug had not been approved by the U.S. Food and Drug Administration (FDA) and was not available for general use. If a physician encountered a case of *P. carinii* pneumonitis, he was required to call the Parasitic Disease Drug Branch of the CDC in Atlanta and provide data to assure the diagnosis was documented or highly likely. If the CDC staff approved, the drug was then sent via commercial airline to the city where the drug was requested. Many of us recall waiting at the airport in the wee hours of the night to grab the precious vials of pentamidine and rush back to the bedside of a critically ill patient to administer the drug intramuscularly. Intravenous administration was thought to be contraindicated at the time. Sadly, on some occasions the drug arrived after the patient died. I am told that some physicians hoarded leftover vials of pentamidine to have it on hand for their next patient while awaiting the CDC delivery circuit. Significant adverse reactions occurred in about 50% of the patients, especially at injection sites.

It was soon recognized that the mortality rate from untreated *P. carinii* pneumonitis in older children and adults with immunodeficiency due to cancer, cancer therapy, organ transplantation, and primary immunodeficiency disorders without treatment approached 100%, considerably greater than the 50% rate in European infants. Thus the efficacy of pentamidine could be ascertained without controlled trials. Several reports of relatively small numbers of cases showed recovery rates of around 45% [55,76,77].

The first comprehensive report on the use of pentamidine in the United States came from Western et al. at the CDC in 1970. They found that 48% of 75 patients recovered with pentamidine therapy [78]. In follow-up, Walzer in 1974 reported that 69 (42%) of the 163 confirmed cases of *P. carinii* pneumonitis reported to the CDC and treated with pentamidine recovered [38].

Adverse effects of intramuscular pentamidine were formidable. Hypotension, hypoglycosemia, nephrotoxicity, neutropenia, thrombocytopenia, rashes,

and injection site reactions often limited the extent of drug use. Of the 404 patients given pentamidine from the CDC, 189 (47%) had 347 adverse reactions [38].

In 1966, Frenkel and colleagues at the University of Kansas Medical Center reported their pivotal study using the cortisone-treated rat model to evaluate drugs for anti-*P. carinii* activity [79]. Their study suggested an approach for the future development of drugs in the treatment and prevention of *P. carinii* pneumonitis. In their study, 6 rats given pyrimethamine plus sulfadiazine survived a mean of 69 days with no organisms found in the lungs, compared to the 6 untreated rats that survived a mean of 46 days and all had cysts in the lungs at autopsy. Later studies of human cases revealed a correlation between drug efficacy for *P. carinii* infection in the rat model and the human disease. In 1966, Rifkind et al. reported the use of pyrimethamine, sulfadiazine, and folinic acid in the treatment of two patients with *P. carinii* pneumonitis [80]. Although both patients died, suggestive evidence of improvement with treatment was noted. Ruskin and Remington in 1967 reported two patients with the pneumonitis treated with the drug combination, but they did not recover [81]. Whisnant and Buckley had a favorable response in the treatment of an infant with the infection [82]. Other reports of individuals or small numbers of cases treated with pyrimethamine and sulfadiazine varied in success rates, with some recoveries and some failures, including the reports by Chobot-Maciejewska et al. [83], Kirby et al. [84], Farinas et al. [85], Kordyasc et al. [86], and Young and De Vita [87].

Because *P. carinii* pneumonitis was endemic in Iranian orphanages, Post and colleagues in 1971 evaluated Fansidar (pyrimethamine and sulfadoxine) for prophylaxis [88]. Between 1968 and 1969 all infants admitted to the orphanage in Shiraz were given either a suspension of the drug orally once weekly or bi-weekly or a placebo. The study comprised 46 infants. Of the 10 infants who died, 3 of the 5 receiving the placebo had *P. carinii* pneumonitis and none of the 5 given the drugs had the pneumonitis.

In 1974, Hughes and colleagues showed that the combination of trimethoprim and sulfamethoxazole was highly effective in the prevention and treatment of *P. carinii* pneumonitis in the corticosteroid-treated rat model [89]. They proceeded to clinical trials, which demonstrated therapeutic efficacy and safety in the treatment of cancer patients with *P. carinii* pneumonitis [90,91]. In a comparative study, trimethoprim-sulfamethoxazole was found to be equal in efficacy but with fewer adverse effects than pentamidine [91]. These studies were soon substantiated with other clinical trials in adults and children with *P. carinii* pneumonitis, including those of Yoshida et al. [92], Winston et al. [93], Lau and Young [94], Chusid et al. [95], Lipson et al. [96], Larter et al. [97], McLennan et al. [98], and Bourgault et al. [99].

Trimethoprim-sulfamethoxazole was approved by the FDA in 1978 for the treatment of *P. carinii* pneumonitis. Because it was readily available in oral and

parenteral formulations, safe, and effective, it soon replaced pentamidine as the drug of first choice for the treatment of the pneumonitis.

By the mid-1970s, several categories of patients were recognized as being at high risk for life-threatening *P. carinii* pneumonitis. For example, predictably, about 15 to 20% of patients with acute lymphocytic leukemia, more than 45% of children with SCIDS, and 9% of organ transplant patients would develop at least one episode. Furthermore, at least one-third of individuals treated for *P. carinii* pneumonitis successfully would have another episode. A means to prevent infections from *P. carinii* pneumonitis was sought. Of the three methods for the prevention of infection—protective isolation, vaccine administration, and antimicrobial drug prophylaxis—chemoprophylaxis offered the greatest promise. Because trimethoprim-sulfamethoxazole provided excellent prophylaxis for *P. carinii* pneumonitis in animals and the drug combination had been used for long periods of time in humans for chronic urinary tract infection or bronchitis, Hughes et al. [100] undertook clinical trials comparing children with cancer who were randomized to trimethoprim-sulfamethoxazole or placebo for the prevention of *P. carinii* pneumonitis. Of the 160 patients enrolled and followed for 2 years in the double-blind study, 21% of the 80 randomized to the placebo and none of the 80 patients given trimethoprim-sulfamethoxazole developed the pneumonitis. Several subsequent clinical trials confirmed the efficacy and safety of trimethoprim-sulfamethoxazole for *P. carinii* pneumonitis prophylaxis in immunocompromised hosts: Harris et al. [101], Wolff et al. [102], Chusid et al. [95], Wilber et al. [103] and Harcy et al. [104]. By the end of the decade, the prophylactic use of trimethoprim-sulfamethoxazole had become a standard of practice in most centers for patients at high risk for *P. carinii* pneumonitis.

G. Organism (1960 to 1980)

By 1960, our understanding of *P. carinii* was based predominantly on the morphological characteristics gleaned from histological studies using the Grocott-Gomori [105] and Giemsa [5] stains. The first electron microscopy studies were done by Wessel and Ricken in 1958 [106] using lung suspensions obtained from rats. While most investigators referred to *P. carinii* as a protozoan, others favored its classification as an ascosporeous yeast.

From 1960 to 1980, several important studies expanded our knowledge of the form and structure of *P. carinii*. Detailed ultrastructural studies of *P. carinii* derived from rats by Barton and Campbell [107], Vavra and Kucera [108], and Vossen et al. [109]; from humans by Bommer [110], Barton and Campbell [111], and Ham et al. [112]; and from other animals by Farrow [113], McConnell [114], and Shively et al. [115] provided data on organelles and cell walls of “cysts,” “trophozoites,” and “sporozoites.” *P. carinii* was first studied by scanning electron microscopy in 1977 by Murphy et al. [116].

In 1972, Kim et al. were the first to show differences among *P. carinii* isolates from humans and rats. Using immunofluorescence techniques, they demonstrated that antisera raised to rat *P. carinii* did not react with human *P. carinii* isolates [117]. The rat antisera reacted avidly with “trophozoites” and “cysts” from rats. The following year, Frenkel, in his excellent review at the NIH Symposium on *P. carinii*, pointed out that although no morphological differences were discernible, the immunological data from the study of Kim et al. established the difference [53]. He suggested that the name *P. jiroveci* be used to designate the human form of the organism.

VII. 1980 to 2002

The history of *P. carinii* can be divided into events before 1981 and events after 1981. Without doubt, this year was a turning point, when *P. carinii* emerged from a relatively obscure organism among human pathogens to a major cause of death in the unrelenting global epidemic of the acquired immunodeficiency syndrome (AIDS). For the two decades after 1980 almost 6000 publications appeared regarding *P. carinii*, compared to around 1000 articles for the two decades prior to 1981.

A. *Pneumocystis carinii* Discovers AIDS (1980 to 2002)

By 1981 most physicians in major medical centers were aware of *P. carinii* as a cause of pneumonitis and the fact that, with rare exception, it occurred only in immunocompromised patients. When a few young men in New York and California were found to have *P. carinii* pneumonitis without any of the known risk factors, astute physicians began to search for an underlying immune defect. In New York, Masur and colleagues [118] encountered 11 men with the pneumonitis, and concomitantly Gottlieb [119], in California, studied 4 similar patients with *P. carinii* pneumonitis. In common were homosexual lifestyles and/or intravenous drug abuse, plus abnormalities in T-lymphocyte function and numbers. Furthermore, suspicion was aroused at the CDC in Atlanta by the commonality of the cases on separate coasts, along with the fact that an increase in requests for pentamidine was occurring. Within the year, AIDS was defined and a new era in human disease began.

It is remarkable that AIDS was discovered and defined with the observations of only 15 cases reported by Masur et al and Gottlieb et al. With these cases, the clinical disease was fully described essentially as we know it two decades and 775,800 cases later. In a sense, *P. carinii* discovered AIDS. Were it not for this opportunistic infection pointing to an immunodeficient host, one wonders how long it would have been before the human immunodeficiency virus (HIV) was discovered, because the virus alone does not produce a recognizable clinical

syndrome. Reflected in these early cases was the fact that adverse reactions to trimethoprim-sulfamethoxazole occurred during therapy for the infection [120]. Because of the unusually high rate of reactions to the drug in common use, requests were arriving at the CDC for pentamidine to be used as alternative therapy.

As the epidemic progressed, the CDC continued to receive more and more orders for pentamidine and the supply in Atlanta diminished. When Myron Schultz at the CDC called May and Baker, Ltd., the only source of pentamidine, to replenish the stock, he learned the company no longer made the drug, had closed the facility for manufacture, and would not be able to resume production. In Atlanta, conferences were held and consultants called. What to do? The CDC decided to purchase all available supplies of pentamidine distributed around the world. This would suffice for the treatment of a few thousand cases. Eventually, other companies (Lyphomed and Fujisawa) took up production of pentamidine, followed by an astonishing increase in the price of the drug.

The role of *P. carinii* as a major infection in AIDS was substantiated as the epidemic expanded. In Jaffee's review [121] of the first 1000 cases of AIDS, 497 (50%) had *P. carinii* pneumonitis. Also, the high frequency of adverse reactions to trimethoprim-sulfamethoxazole was apparent from reports of around 45% of cases being so affected [122]. Unfortunately, AIDS patients also suffered equally high or higher adverse effects from pentamidine, the only other drug available for the treatment of sulfonamide-sensitive patients. New drugs were desperately needed. By mid-1983, over 2000 cases of AIDS had been reported to the CDC and 743 cases of AIDS plus *P. carinii* pneumonitis [123,124].

B. Treatment and Prevention (1980 to 2002)

The demand for new alternative drugs for the treatment and prevention of HIV-infected patients led to the development of several effective and relatively safe interventions. Five anti-*P. carinii* regimens reached clinical trials with success and are now currently in use with FDA approval (three for *P. carinii* indication). These include dapsone with and without trimethoprim; aerosolized pentamidine; trimetrexate-leucovorin; atovaquone; and clindamycin plus primaquine. Pyrimethamine-sulfadoxine has found limited use for the prophylaxis of *Toxoplasma gondii* and *P. carinii* infections.

In 1984 Hughes et al. reported the anti-*P. carinii* activity of dapsone (diaminodiphenylsulfone) in the rat model and demonstrated synergistic effects with trimethoprim [125]. Clinical trials in AIDS patients soon confirmed the therapeutic effects [126], the importance of synergy [127], and that dapsone was effective for prophylaxis in high-risk AIDS patients [128–131].

A keen clinical observation by a prepared mind has often led to discovery. Such was the case in 1985, when MacFadden et al. noted a seemingly paradoxical

phenomenon [132]. When a patient with severe *P. carinii* pneumonitis and AIDS was inadvertently given a corticosteroid drug systemically, a striking clinical improvement followed. In the same year, Foltzer et al. made a similar observation [133]. Soon other anecdotal cases were reported describing improvement in the clinical course related to corticosteroid administration. By 1990, five controlled trials evaluating adjunctive corticosteroid therapy were reviewed by an NIH–University of California Expert Panel [134]. The panel concluded that adjunctive corticosteroid therapy can reduce the likelihood of death, respiratory failure, or deterioration of oxygenation in patients with moderate-to-severe *P. carinii* pneumonitis. This scheme became incorporated into practice guidelines, although some subsequent papers have questioned the overall value of this therapy. It is of historical interest that the classic paper of Kass et al. in 1950 described the use of a corticotropic hormone in the treatment of patients with severe pneumococcal pneumonitis [135]. All of the patients improved despite persistently positive blood cultures. Exactly how corticosteroid drugs that are clearly responsible for provoking *P. carinii* pneumonitis can also evoke improvement in respiratory function and survival has not been fully delineated.

In 1987, a novel approach was taken for the development of a new anti-*P. carinii* drug. Allegra [136] and Queener [137] found trimetrexate, a lipid-soluble derivative of methotrexate, to be a potent inhibitor of *P. carinii* dihydrofolate reductase in vitro. In clinical trials, leucovorin was added to protect the human dihydrofolate reductase. Allegra et al. [138] and Sattler et al. [139] found trimetrexate-leucovorin to be therapeutically effective in complicated cases of *P. carinii* pneumonitis; however, relapses occurred in up to 60% of cases within 3 months of treatment [140]. This drug was approved by the FDA in 1994 for the treatment of patients with *P. carinii* pneumonitis not responding to or unable to take the primary therapy drugs.

Because, with rare exception, *P. carinii* infection is limited to the lungs, the idea of delivery of the drug by aerosol inhalation to avoid systemic toxicity seemed reasonable. In 1986, Bernard, et al. [141], and in 1987, Debs et al. [142] and Girard et al. [143] showed that aerosolized pentamidine effectively treated and prevented *P. carinii* infection in rats. Clinical trials by Conte et al. [144] and Montgomery et al. [145] produced favorable results in the treatment of mild to moderate cases of the pneumonitis in AIDS patients. However, in later studies, Conte et al. [146] and Soo Hoo et al. [147] in 1990 found unacceptable failure rates of 45% or greater. Subsequent studies showed aerosolized pentamidine to be effective and safe for the prevention of *P. carinii* pneumonitis in AIDS patients [148,149]. In 1989, aerosolized pentamidine was approved by the FDA for prophylaxis against *P. carinii* pneumonitis.

In 1990, Hughes et al. found that a hydroxynaphthoquinone (566C80, later named atovaquone) was highly active as an anti-*P. carinii* agent in the rat model

[150]. A pilot study in AIDS patients by Falloon et al. suggested its efficacy and safety for the treatment of the pneumonitis [151]. Subsequently, a randomized controlled double-blind study by Hughes et al. compared atovaquone tablets with trimethoprim-sulfamethoxazole for the treatment of mild to moderate cases of *P. carinii* pneumonitis [152]. Overall success rates were similar when balanced by a more effective therapeutic effect with trimethoprim-sulfamethoxazole but greater safety with atovaquone. Later, the formulation was improved with a suspension, providing greater bioavailability. Atovaquone was approved by the FDA in 1995 for the treatment of mild to moderate cases. El Sadr et al. reported a randomized trial in more than a thousand HIV-infected patients at risk for *P. carinii* where atovaquone and dapsone were found to be equally effective as prophylactic regimens [131]. Atovaquone is safe in long-term prophylaxis for HIV-infected infants and children [153].

Queener et al. [154] in 1988 and Smith et al. [155] in 1991 found the combination of clindamycin and primaquine to have anti-*P. carinii* activity in vitro and in the rat model. Clinical evaluations in small groups of patients suggest that the drug combination is effective and relatively safe [156–161]. Clindamycin and primaquine did not prove successful in prophylaxis [162].

In the early years of the AIDS epidemic, physicians were slow to apply chemoprophylaxis for *P. carinii* pneumonitis despite the fact that trimethoprim-sulfamethoxazole prophylaxis was well established for patients with cancer, congenital immunodeficiency disorders, and organ transplants. Only one study compared randomized AIDS patients receiving trimethoprim-sulfamethoxazole to a comparable group receiving no prophylaxis. In this 1989 study, Fischl et al. [163] found that 52% of the control patients developed *P. carinii* pneumonitis and none of those given trimethoprim-sulfamethoxazole prophylaxis had the infection. A significant factor in the implementation of effective prophylaxis in HIV-infected patients at high risk was the publication of “Guidelines for the Prophylaxis against *Pneumocystis carinii* Pneumonitis for Persons Infected with HIV” by the CDC in the *Morbidity and Mortality Weekly Report* on June 16, 1989 [164]—some 8 years after the epidemic was recognized. This report provided an “official” statement that set the standard of practice and was followed by a noticeable impact on prophylaxis usage. As a consequence of chemoprophylaxis in high-risk AIDS patients—such as those adults with CD4 lymphocyte counts below 200/cm³—the incidence of *P. carinii* pneumonitis after the diagnosis of HIV infection dropped significantly. A CDC analysis from 1990 to 1999 of 19,081 AIDS patients at high risk showed that the overall incidence of the pneumonitis had decreased to about 5.2% [165].

As highly active antiretroviral therapy (HAART) greatly improved the survival and immune status of AIDS patients, those once at high risk for *P. carinii* pneumonitis were able to reconstitute their CD4 lymphocyte counts to levels that

allowed many to have prophylaxis safely withdrawn [166–170]. Thus prophylaxis provided a bridge to long-term survival by protecting against *P. carinii* pneumonitis, while HAART affected viral load and immune reconstitution.

C. Organisms and Host Interaction (1980 to 2002)

Most of the significant advances related to *P. carinii* in the last decade have been at the cellular and molecular levels to elucidate the basic structure, organization, and function of the organism and how the host responds to infection. That is evident in this volume, where 15 of the 24 chapters are devoted to topics in the basic science of *P. carinii* infection. Because background information in these chapters will provide historical perspective, a detailed overview is not included here.

The backbone of recent scientific advances has been the molecular genetic information gained by analysis of the *P. carinii* genome using powerful tools of restriction enzyme analysis, electrophoretic karyotyping, and the cloning and sequencing of DNA segments. Genetic diversity of *P. carinii*, as with other organisms, brings new challenges to scientists but also provides opportunities to expand epidemiology by molecular typing of isolates. Comparison of macromolecular sequences that share a common evolutionary relationship has given insight into the taxonomical position of *P. carinii* [171,172] and at the same time raised new, unanswerable questions, because the existing definitions of fungi, protozoa, alga, bacteria, and so on have not been based solely on genetic analysis. The question of concern is how to define fungi and protozoa by genetic classification. Nevertheless, the important issue is the value of these admirable studies, which have advanced our knowledge of the organism.

In 1988 Wakefield et al. [173] reported the cloning of *P. carinii* DNA and subsequently applied the use of the polymerase chain reaction (PCR) to amplify sequences for the detection and quantification of components from rat, human, and environmental sources. Thus the development of a clinically applicable diagnostic system was launched [174,175].

Methods to detect *P. carinii* DNA by amplification of DNA by PCR provided powerful tools for the study of *P. carinii* infection an order of magnitude greater than histological and serological methods. Peters et al. demonstrated that numbers of *P. carinii* organisms could be calibrated with amplification and hybridization signals to show the detection of *P. carinii* to a lower limit of one to two organisms [176]. They were able to determine the number of organisms in a lung specimen that corresponds to *P. carinii* pneumonitis or to a subclinical pulmonary colonization.

Despite the success of several investigators from 1980 to 2002 in developing an in vitro culture system that maintains or propagates *P. carinii* for varying

periods of time, no satisfactory method for cultivation of the organism has evolved that will allow indefinite growth and perpetuation in vitro [177–183].

D. Animal Models (1980 to 2002)

During the 1980–2002 period, research utilizing animal models flourished. Walzer and colleagues in Cincinnati expanded our knowledge of host-parasite interactions and the pathogenesis of *P. carinii* pneumonitis through the use of the rat and mouse models [184–191]. They found antibody responses to *P. carinii* among different strains of normal and athymic mice to be mainly IgG- and T cell-dependent and that antibodies are not important to susceptibility and resistance [188]. They also found that rat and human species have antigenic characteristics that are shared as well as species-specific [189]; that the infection affects the permeability of alveolar-capillary membranes [187]; that the extent of pneumonitis is related to the quantity of cysts in the lungs [185]; and that the administration of the major surface glycoprotein (MSG) elicits an antibody response [191].

Cesarean-obtained and barrier-sustained “germ free” rats gave Hughes and colleagues [192][193] the opportunity to demonstrate the natural mode of transmission from animal to animal by the airborne route and the inability to transmit the organism via water, soil, and food.

In 1983 Kernbaum et al. [194] first showed evidence of increased catabolism of surfactant, the phospholipid complex essential for normal lung compliance in *P. carinii* – infected rats. Sheehan et al. [195], Stokes [196], Pesanti [197], Eijking [198], Rice [199], and others have extended the studies on surfactant to further elucidate the effect of *P. carinii* infection on the mechanism of respiration. Eijking [200] and Hughes et al. [201] demonstrated improved pulmonary gas exchange and survival rates, respectively, of rats administered replacement of surfactant phospholipids by aerosol.

Recently studies by Beers et al. [202] have given further insight into the role of *P. carinii* on surfactant protein B expression in infected mice. Wright and Gigliotti's [203] elegant studies in the mouse model suggest that it is the inflammatory process of *P. carinii* infection that accounts for disruption of surfactant. Maher et al. [204] and Linke et al. [205] showed that surfactant-deficient mice have increased susceptibility to *P. carinii* infection.

Based on animal studies, the use of synthetic surfactant in the treatment of *P. carinii* has been implemented individual cases of *P. carinii* pneumonitis in humans [206][207].

In 1988, Bartlett et al. [208] devised a modification of the traditional corticosteroid-treated rat model by administering *P. carinii* organisms intratracheally or transtracheally into immunosuppressed rats. This has made possible experimental manipulation not possible with the naturally occurring infection. In 1990, Shellito

et al. [209] proposed a new model in mice by selectively depleting helper T lymphocytes with the use of anti-CD4 lymphocyte antibodies. In the same year, Harmsen and colleagues [210] demonstrated in mice the requirement for the CD4 lymphocytes in host resistance to this organism. Also, Fisher et al. [211] showed a specific T-cell response in mice to a *P. carinii* surface antigen (GP 120) after immunization and natural infection. Chen et al. [212] used SCID mice to slow the development of *P. carinii* pneumonitis and also to achieve resolution of the infection with immune reconstitution. These authors [213] found that latency is not the inevitable outcome of *P. carinii* infection in mice, and Vargas et al. [214] made similar observations in rats. Oz et al. studied the effects of CD₄₀ ligand and other immunomodulators on the rat model infected with the organism [215].

In the second edition of *Pneumocystis carinii Pneumonia*, in 1994, the reviews of “Animal Models” by Armstrong and Cushion [216] and “New Animal Models for *Pneumocystis carinii* Research: Immunodeficient Mice” by Sidmon and Roths [217] appeared extensive and masterful descriptions of advances in this field that are far more comprehensive than it is possible to present in this historical overview.

VIII. Conclusions

Unknown at the beginning of the twentieth century, *P. carinii* is now recognized as one of the most prevalent microbes infecting humans and lower mammals. Its distribution is worldwide, comprising all continents and in all climes. Unique among all infections is its remarkable tropism for the alveolar spaces and limitation to the lungs even in fatal cases. The majority of humans and rats acquire the infection early in life. In immunocompetent host, the infection is adequately controlled by the host, and few if any signs or symptoms of illness develop. In the severely immunocompromised host, fatal infection occurs. Recent studies by Vargas et al. now suggest that mild, self-limited respiratory illnesses due to *P. carinii* may occur in many normal infants [218].

Out of the necessity to help our patients survive, the early years of research sought methods for diagnosis, treatment, and prevention of *P. carinii* pneumonitis, moving pragmatically to these ends. The beginning of the twenty-first century offers great opportunities to further elucidate the nature of the organism and the disease it causes. It is reasonable to expect that the clinical spectrum will come to include newly recognized expressions of disease of a self-limiting nature in otherwise normal individuals; that the organism's natural habitat will be determined; that improved drugs for the prevention and treatment of *P. carinii* pneumonitis will be developed; and that the organism will be perpetuated through its complete life cycle and indefinitely with an in vitro culture system. The third edition of *Pneumocystis carinii Pneumonia* is the first step of the bridge into the new century and reflects the promise for remarkable advances.

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2

Nomenclature and Genetic Variation of *Pneumocystis*

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I. Introduction

Studies during the last decade have revealed that the genus *Pneumocystis* contains many distinct organisms. The genetic and functional diversity exhibited by these organisms indicate that they are evolutionary species. While the number of *Pneumocystis* species is not known precisely, it is highly probable that every species of mammal has at least one.

With improved understanding of diversity in the genus comes a need for nomenclature changes. This chapter explains the rationale for the changes that have already been made and provides guidelines for assigning new species names.

A. History

The first reported observation of *Pneumocystis* appeared in 1909 [1]. While studying mammals infected by New World trypanosomes, Carlos Chagas noticed cyst-like organisms in their lungs. He suggested that these cysts were reproductive forms of the trypanosome. However, Antonio Carini observed a similar microbe in the lungs of Old World animals, namely European sewer rats [2]. In 1912, Delanoë and Delanoë examined microscope slides provided by Carini and concluded that they contained a microbe unrelated to trypanosomes. Accordingly,

they erected a new genus, *Pneumocystis*, and named the organism in rats *Pneumocystis carinii*, in honor of Carini [3].

A microbe from the genus *Pneumocystis* was implicated in pneumonia in humans in 1942 by van der Meer and Brug, who noted characteristic “honey-comb” patterns in alveoli of children with plasma cell interstitial pneumonia [4]. In 1952, Vanek and Jirovec reported *Pneumocystis* to be the cause of interstitial plasmacellular pneumonia in neonates [5]. A more detailed history is presented in Chapter 1.

II. *Pneumocystis* Is a Fungus

For seven decades, most investigators thought *Pneumocystis* organisms to be protozoans because they do not look much like fungi, fail to grow much in culture, and are not eliminated from patients by treatment with the antifungal agents amphotericin B and ketoconazole. By contrast, drugs, such as trimethoprim-sulfamethoxazole and pentamidine, which are often useful in treating protozoan infections, are also active against *Pneumocystis*.

The protozoan hypothesis was not universally accepted. Circa 1970, fungus-like morphological features were noted [6]. Furthermore, trimethoprim-sulfamethoxazole inhibits biosynthetic pathways common to fungi and protozoa and thus is not a good discriminator. Nevertheless, the protozoan hypothesis held sway until it was found that the sequence of the 18S ribosomal RNA (rRNA) gene of *Pneumocystis* from rats is much more like those of fungi, such as *Saccharomyces cerevisiae*, *Candida albicans*, *Neurospora crassa*, and *Cryptococcus neoformans* than to the 18S rRNA genes in protozoa [7,8]. Subsequent comparisons of the 18S rRNA sequences from 35 fungi placed *Pneumocystis* on a phylogenetic branch near the ascomycetes [9]. Data from other genes also indicated that *Pneumocystis* is most closely related to ascomycetes (reviewed in Refs. 10 to 13).

The number of gene sequences available increased dramatically with the advent of projects to sequence the genomes of several fungi, including *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces pombe*, and *Pneumocystis* from rats. The genomes of the first three species have been completely sequenced. More than 1400 *Pneumocystis* genes have been partially sequenced. Nearly all (97%) of these 1400 genes are most similar to an orthologous gene in one fungus or another (orthologous genes are genes that exist in different species but are the same gene by descent) (see <http://biology.uky.edu/Pc/>). Sixty-five percent of *Pneumocystis* genes are most similar to an orthologue from the well-studied ascomycete *S. pombe*. These data, along with those from rRNA, place *Pneumocystis* in the phylum Ascomycota. The alternative view, that these DNA sequence similarities may be due to so-called horizontal transfer events between *Pneumocystis* and one or more ascomycetes, seems untenable, given the very large number of genes that show high similarity to those of ascomycetes.

The clear genealogical resemblance to ascomycetes stands in stark contrast to phenotypic data (reviewed in Refs. 10 to 12). As mentioned above, *Pneumocystis* organisms do not proliferate much in culture, while the ascomycetes *S. cerevisiae*, *C. albicans*, and *S. pombe* are famed for their robust growth in media containing the simplest of ingredients. In addition, *Pneumocystis* organisms lack the sturdy cell wall typical of fungi. Finally, *Pneumocystis* organisms lack detectable amounts of the characteristic fungal sterol ergosterol and contain a large amount of cholesterol in the cell membrane instead [14–16]. This difference explains the relative resistance of *Pneumocystis* to amphotericin and ketoconazole, both of which are particularly toxic to fungi because they are rich in ergosterol. The source of the cholesterol in *Pneumocystis* is not clear, but it could be obtained from the mammalian host. Small amounts of ergosterol may be made. Both amphotericin B and ketoconazole have been shown to be capable of reducing the viability of *Pneumocystis* organisms maintained in culture, suggesting that biosynthesis of ergosterol or something like it is occurring and is needed [18]. Recent data have shown that the genome of *Pneumocystis* contains a gene that encodes S-adenosyl-methionine delta-24-sterol-c-methyltransferase, suggesting that *Pneumocystis* can make sterols [17]. Indeed, sterols other than cholesterol have been found in *Pneumocystis* organisms, and one of these may be unique to this genus [16]. Biochemical studies support this view. The *P. carinii* S-adenosyl-methionine delta-24-sterol-c-methyltransferase is unusual with respect to the kinds of substrates it acts upon [17].

III. Is *Pneumocystis* One Species or Many?

The fungal nature of *Pneumocystis* was established using organisms obtained from laboratory rats. When DNA sequences in *Pneumocystis* organisms from other host species were examined, it became immediately apparent that *Pneumocystis* organisms are not all the same, suggesting that the genus *Pneumocystis* contains more than one species. However, while speciation is always accompanied by genetic change, members of the same species can and do exhibit genetic differences. Therefore the question became: “Is the sequence variation exhibited by *Pneumocystis* organisms indicative of different species?” In addressing this question, it is useful to first outline the “species problem” in general.

This problem entails two questions: “What are species?” “How can they be recognized?” There is no shortage of proposed answers to these questions. In a recent review, Mayden lists 20 different species concepts (see Table 1) [19]. The biological species concept (BSC) is probably the one that is familiar to the largest number of biologists. It defines species as groups of interbreeding natural populations that are reproductively isolated from other such groups [20,21]. An advantage of the BSC is that it provides an objective test for recognizing members of the same species, no matter how different they may appear. If two organisms

Table 1 Species Concepts

Species concepts	Species concepts (cont.)
Biological species concept (BSC) ^a	Composite species concept (CpSC)
Morphological species concept (MSC)	Genealogical concordance concept (GCC)
Phylogenetic species concept (PSC) ^{a,b}	Genotypic cluster definition (GCD)
Evolutionary species concept (ESC) ^a	Internodal species concept (ISC)
Genetic species concept (GSC) ^a	Cohesion species concept (CSC) ^a
Hennigian species concept (HSC) ^a	Nondimensional species concept (NdSC)
Ecological species concept (EcSC) ^a	Phenetic species concept (PhSC)
Recognition species concept (RSC) ^a	Polythetic species concept (PtSC)
Reproductive competition concept (RCC) ^a	Successional species concept (SSC)
Agamospecies concept (ASC)	Taxonomic species concept (TSC)
Cladistic species concept (CLSC)	

Sources: Adapted from Refs. 19 and 65.

^a Concepts whose defining characteristic is a biological process (for example, competition or reproduction) shared among members of a species (65).

^b Diagnosable version of the PSC. There are two other versions of this concept, including the monophyly version and the diagnosable/monophyly version, neither of which solely uses biological processes.

can mate productively, they are members of the same species. Conversely, if two organisms cannot mate productively, they are members of different species, no matter how similar they may appear. Unfortunately, the BSC is not useful for distinguishing species in cases such as *Pneumocystis*, where mating tests cannot be conducted. Another well-known concept is the morphological species concept (MSC), which uses morphology as the sole criterion for distinguishing species. While nearly all microbes have been assigned to species based on this concept, it cannot discern variation that is not accompanied by obvious morphological difference, as is often the case for members of the genus *Pneumocystis*.

The inapplicability of these two popular and well-understood species concepts, combined with the difficulty of working with a fastidious microbe, discouraged efforts on the taxonomic front. While some morphological differences were described, [22–26], all *Pneumocystis* organisms seemed to be the essentially the same, although not necessarily identical. It is not surprising that, prior to the advent of gene sequencing, proposals to designate more than one *Pneumocystis* species were rare. The few new species names that were proposed fell rapidly into disuse [27,28]. Even after gene sequencing revealed the diversity within the genus, uncertainty dominated the debate regarding designation of multiple species.

Because the BSC could be not applied and morphology was not definitive, new species names were not adopted at first. Instead, diversity was recognized via the taxonomic device of designating “special forms” (see *The International*

Code of Botanical Nomenclature, Chapter 1, Article 4, Note 3. The code is available on the Internet at <http://www.bgbm.fuberlin.de/iapt/nomenclature/Code/SaintLouis/0001ICSLContents.htm>). Special forms are two or more fungal parasites that might be the same species based on morphology but are distinguishable by their adaptation to different hosts. The “special form” designator fits *Pneumocystis* perfectly because, although this microbe infects a broad range of mammals, gene sequence comparisons of *Pneumocystis* from different host species had shown that each host species has its own type of *Pneumocystis* [11,12,29,30,33]. Furthermore, *Pneumocystis* from a given host species appear to be unable to proliferate in other host species [29]. In one set of experiments, organisms taken from ferrets failed to grow in severe combined immune deficiency (SCID) mice [34]. Other laboratories have generally found that *Pneumocystis* from one host species do not proliferate when introduced into other host species [35–40]. Some studies have reported cases of apparent transfer between host species, but these cases are not convincing because they lack proof that the infection was caused by the microbe transferred and not by *Pneumocystis* organisms that were already in the recipient animal.

Given the debate surrounding species, it seemed both practical and prudent, until recently, to use the “special form” method to distinguish different members of the genus. To accomplish this, a special form name was appended to the genus-species binomial. Accordingly, *P. carinii* from rats was renamed *P. carinii* f. sp. *carinii*. The special form names for other members of the genus are listed in the section on nomenclature below.

In the years following the introduction of the “special form” system, progress in understanding the phylogenetics of *Pneumocystis* was aided both by the availability of additional sequence data and the appearance of new taxonomic and phylogenetic tools, which were developed because the factors that make it difficult to determine the significance of diversity among *Pneumocystis* organisms are not unique to this genus. While the designation of special forms has been useful as a temporary device, the trinomial nomenclature suffers from a serious disadvantage compared to a binomial system. Two organisms that share a genus name but each have their own species names are immediately recognizable as relatives that must be regarded as stable, distinct biological entities. By contrast, the significance of the “special form” designation must be defined every time one uses a tripartite name. Hence, the arcane nature of the tripartite name deprives the broad audience of individuals interested in *Pneumocystis* of vital information. Proper recognition of the differences among genus members is important for several reasons, not the least of which is that it will stimulate more research and help in assessing the relationships between human health and findings obtained by studies on animal-borne members of the genus.

An additional impetus to phase out special forms and replace them with new species names comes from an improved appreciation of the utility of the phylogenetic species concept [41]. This concept is based on the broader evolution-

ary species concept (ESC), which defines a species as a single lineage of ancestor-descendant populations that maintains its identity from other such groups and has its own independent evolutionary history [42]. The ESC is highly utilitarian because it does not specify the means by which a lineage of ancestor-descendant populations (what we will call an *evolutionary species*) is to be recognized. Thus, if morphology and mating are not applicable criteria, as is often the case with *Pneumocystis* organisms, this situation does not preclude recognition of evolutionary species. The ESC does not forbid basing species on gene sequence data because a specific collection of gene sequences will uniquely accumulate in an evolutionary species. Related evolutionary species—i.e., those from a common ancestor—will have related collections of gene sequences due to the combined effects of lineal descent, random variation, and selection. Because these same genetic processes generate the morphological, biochemical, and other differences that are traditionally used to recognize species, the ESC can be viewed as the preeminent definition of a species, with all other concepts being secondary to it [19].

The perspective provided by the ESC points the way forward for revising the classification and nomenclature applied to the genus *Pneumocystis*. It has been clear for some time that the genomes of *Pneumocystis* organisms from different host species differ to a degree that exceeds that seen among species within other fungal genera. These differences have been described previously [10–12,29,30,43]. To illustrate the point, a few examples are presented here. The mitochondrial large subunit ribosomal RNA (mt LSU rRNA) genes of *Pneumocystis* from mouse and rat are approximately 10% different, and even greater differences separate rodent and primate *Pneumocystis*. It has been shown that the *Pneumocystis* from 18 primate species varied from 2 to 28% at the mt LSU rRNA locus [33]. These values are very large compared to divergences observed in the genus *Saccharomyces* (members vary by approximately 1% at this locus), for example. Yet the *Saccharomyces* organisms in question are bonafide biological species. Another example of high divergence is shown in Table 2, which contains genetic distances calculated by aligning DNA sequences of 18S rRNA genes. *Pneumocystis* isolated from rat and human are divergent from one another by 4% [8,44]. This is approximately twice the 18S rRNA divergence exhibited by *Candida albicans* and *Candida tropicalis* (1.6%). In addition, it is nearly five times greater than the divergence between *S. cerevisiae* and *Saccharomyces rosei* (0.7%). These data indicate that either there are multiple species in the genus *Pneumocystis* or members within a hypothetical single species of *Pneumocystis* diverge to an extraordinary degree compared to other fungal genera.

Not only do different hosts harbor distinct *Pneumocystis* species, but the same host can have more than one as well. Studies several years ago showed two distinct *Pneumocystis* rRNA gene sequences in a single ferret, which implies that there are two different species of *Pneumocystis* in this host species [45–47].

Table 2 Divergence of 18S Ribosomal DNA Sequences: *Pneumocystis* Species Compared to Species in the Genera *Saccharomyces* and *Candida*

Species compared ^a	Percent divergence ^b	Number of nucleotide differences ^c
<i>P. carinii</i> and <i>P. jiroveci</i>	3.8	17
<i>S. cerevisiae</i> and <i>C. albicans</i>	6.1	27
<i>C. albicans</i> and <i>C. parapsilosis</i>	2.3	10
<i>C. albicans</i> and <i>C. tropicalis</i>	1.6	7
<i>C. parapsilosis</i> and <i>C. tropicalis</i>	1.6	7
<i>C. parapsilosis</i> and <i>C. viswanathii</i>	1.3	6
<i>C. tropicalis</i> and <i>C. viswanathii</i>	0.9	4
<i>S. cerevisiae</i> and <i>S. rosei</i>	0.7	3

^a The following 18S ribosomal gene sequences were used: *Saccharomyces cerevisiae*, strain S288C (accession number (acc. no.) U53879); *Saccharomyces rosei* (*Torulaspora delbrueckii*), strain MUCL27816 (acc. no. X53496); *Candida albicans*, strain MUCL29800 (acc. no. E15168); *Candida parapsilosis*, strain ATCC 22019 (acc. no. AYO55855); *Candida tropicalis* (acc. no. AF290988); *Candida viswanathii*, strain JCM 9567 (acc. no. AB013589), and *Pneumocystis* spp. (44).

^b The 3' terminal 444 nucleotide sites of 18S rDNA were aligned and distances were computed for each pairwise comparison and converted into percentages. Percent divergences were calculated using p-distance (i.e., the proportion of nucleotide sites at which the two sequences compared are different and is calculated by dividing the number of nucleotide differences by the total compared nucleotide sites) multiplied by 100.

^c The number of nucleotide substitutions of each pairwise comparison.

Even prior to this observation, pulse field gel electrophoresis of *Pneumocystis* chromosomes had shown that rat has two genetically distinct organisms [48]. One of these was initially referred as “prototype” *P. carinii*. This species was validly named *P. carinii* in 1999 [49]. The other organism in rats was initially called “variant.” When the “special form” system was introduced, it became *P. carinii* sp. f. *ratti*. It has since been renamed *Pneumocystis wakefieldiae* [48]. The decision to assign this new species name is base on a large body of gene sequence evidence. *P. wakefieldiae* genes that differ from *P. carinii* orthologues include thymidylate synthase, TATA-box binding protein, the small and large mitochondrial ribosomal RNAs, the α subunit of the G protein, and nuclear ribosomal RNA. The *P. wakefieldiae* gene encoding nuclear ribosomal RNA also lacks the group 1 intron that is present in the gene in *P. carinii* [47,50–53]. In addition, *P. carinii* and *P. wakefieldiae* differ with respect to the sequences and structures of genes encoding the major surface glycoprotein (MSG) genes [48,54].

An example of the extensive genetic difference between *P. carinii* and *P. wakefieldiae* is illustrated in Figure 1, which shows alignments of thymidylate synthase (TS) genes [55]. Nucleotide nonidentities are located both in the introns and in the coding regions. Thirteen substitutions occur in the coding regions.

PJ GATTATACAG GTCAAGGTGT TGATCAGCTA GCCAATATTA [40]
 PO A... ..C..A... ..C..
 PF A....C... ..T...GT..
 PC A.....TT. .A..... ..T... ..
 PW A..... .A..... ..T... ..
 PM A.....T... .A..... ..T... ..
 CA C. T. T... CAAG.CG..
 SC ..C.....T. .A.....A. T.G AAAC.GG..

PJ TTCATAAAAT TCAAACATCT CCATATGATC GTCGACTAAT [80]
 PO ..G.A..... ..G..... ..T..
 PF A.GT..T..C ..T..... ..AT..
 PC .C..... A.G.....A C..
 PW ..A..... A.....A T..
 PM A.G.....A C.....
 CA ..A.....T. GA....AA. A .AA..A.T..
 SC .A.....C. AA....AA. ..C..C...A .AA..A.C..

PJ CCTTATGGCA TGGAACCCAG CAGATCTAAA AAAGATGGCA [120]
 PO T.....C A CT.... ..A.....G
 PF CA... T.... T.... ..A.....
 PC A...TCA... T..T. T..G. ...A.....
 PW T...TCA... T..T. T..G. ...A.....
 PM A...TCA... T..TT T..G. ...A.....
 CA .A.GTCT..T T..GC T.TGC T..A.....C
 SC TA.G.GT..C T...T.TG. C..A.....T

PJ TTACCTCCAT GTCACATGTT TTGTCAATTT TATGTTTCATA [160]
 PO ..G..... .C..T.... C..... ..C.....G
 PF C.T..... ..G.... ..C.....
 PC G. .C..T.... G....
 PW G. .C..... ..
 PM ..G.....G. .C.....C.....
 CA ..G..... ..G.T.. C.....GA..T
 SC ..G..G.... .C..T..T... ..CA..G..C CAG.T

PJ TACCA----- -----CC [200]
 PO----- -----T..
 PF A...G----- -----A
 PC----- -----T..
 PW ..T...----- -----T..
 PM----- -----T..
 CA .C..TACATC TGTGCCGGAC CCAAATAATC CTAAACAAG.
 SC .C..C----- -----AAAGAA--GGAGAAGG

PJ AAATGGCGGT CGACGTGAAC TTTCATGT [228]
 PO GG....TC.. .A..... .C.....C
 PF G....T.A. AA.GC.A.G.
 PC G...AA.CAC C..... .C.....
 PW G...ATCAC C..... ..
 PM G...A..CAC C..... .C.....
 CA T..AACG.CC AA..CAA..T .A..T..
 SC ---TC.... AAG.CCCGC. C

Intron B

```

PJ  GTATTCTTCCTTACTAAATTAATATTACCTCTTAATATAACTTAG   [45]
PF  .C.CATA.TT...T.C.T..GTC...T.-.A.....ATCA....
PC  .C..A..CTT...TC..T.C.....C.TTG...T..CT.T....
PW  ....AT..TT...TC..T.C.....C.TTG...T..CT.TA....
PM  ....A...TT...TC..T.C.....C.TTG...T..CT.T....
PO  .....T.CTA...ACTG.AAG.A..C-...-A.....G...T....

```

Intron C

```

PJ  GTAATACATCTTAAA-TCCTATATAT--AA---CCTAATATT--TACAAA-ATATATAG   [57]
PF  ....G-----GGT-..T.T.T.TA--..TT--...T.....TTTCC.....
PC  ....TTCA.ATTTAA.....GA..A--..AT.A.G-CT.....CC.C.....
PW  ....GTT.A.ATTTAAT..G.....GGT.A--..ATTAGAACT.....CT.....
PM  ....TT.A.ATTTAA...G.....GGT.A--..ATGA..-CT.....CT.....
PO  ....C.-----TTTGC..AG.T.T.--TAGAGAA.TAC...-ATA.TT-.CG..C..

```

Figure 2 Nucleotide alignment of thymidylate synthase introns B and C. The length of each alignment is shown to the right of the top line for each intron. Abbreviations for organisms: PJ, *Pneumocystis jiroveci*; PF, *P. carinii* f. sp. *mustalae*; PC, *Pneumocystis carinii*; PW, *Pneumocystis wakefieldae* (formerly *P. carinii* f. sp. *ratti*, also known as “variant”); PM, *P. murina* (formerly *P. carinii* f. sp. *muris*) PO, *P. carinii* f. sp. *oryctolagi*; CA, *Candida albicans*; SC, *Saccharomyces cerevisiae*. Genbank accession numbers for sequences were *P. carinii* (no. M25415), *Candida albicans* (no. J04230), and *Saccharomyces cerevisiae* (no. J02706). *P. carinii* f. sp. *oryctolagi* sequence was from [55,55a].

Differences are of two kinds: nucleotide substitutions and length polymorphisms (see Fig. 2). Figure 3 shows deduced TS protein sequences. These also vary, showing that nucleotide substitutions are not all silent.

A powerful way to depict the variation described above is through construction of gene trees. A gene trees displays the relatedness of a set of orthologous

←

Figure 1 Alignment of deduced thymidylate synthase amino acid sequences. Amino acid sequences were deduced from the nucleotide sequences shown in Figure 1. Dots indicate identity and dashes indicate gaps that were introduced for maximal alignment. Abbreviations for organisms: PJ, *Pneumocystis jiroveci*; PF, *P. carinii* f. sp. *mustalae*; PC, *Pneumocystis carinii*; PW, *Pneumocystis wakefieldae* (formerly *P. carinii* f. sp. *ratti*, also known as “variant”); PM, *P. murina* (formerly *P. carinii* f. sp. *muris*) PO, *P. carinii* f. sp. *oryctolagi*; CA, *Candida albicans*; SC, *Saccharomyces cerevisiae*. Genbank accession numbers were *P. carinii* (no. M25415), *Candida albicans* (no. J04230), and *Saccharomyces cerevisiae* (no. J02706). *P. carinii* f. sp. *oryctolagi* sequence was from [55,55a].


```

PJ DYTGGQGV DQL ANIIHKIQTS PYDRRLILMA WNPADLKK [38]
PO .....E.....T....
PF ....K.....G.....R...H..T.....
PC N.I.....R.....S.....E.
PW N.....N.....S.....E.
PM N.I.....R.....S...S..E.
CA .....F... QDV.N.LK.N .....I.MS. ...P.FA.
SC .....I... KQV...LK.N .....I.MS. ....FD.

PJ MALPPCHMFC QFYVHIP--- -----PN GGRRELSC [76]
PO .....V.--- -----SD .RQ.....
PF .....K.--- -----Q. .DKAK...
PC .....--- -----S. NH.P....
PW .....S--- -----S. DH.P....
PM .....I.. -----S. SH.P....
CA .....V.. ....NF.TSV PDPNNPKQAK TAKPK...
SC .....I.S .....SF.--- ---KE-GEG- S.KPR...

```

Figure 3 Alignment of deduced thymidylate synthase amino acid sequences. Amino acid sequences were deduced from the nucleotide sequences shown in Figure 1. Dots indicate identity and dashes indicate gaps that were introduced for maximal alignment. Abbreviations for organisms: PJ, *Pneumocystis jiroveci*; PF, *P. carinii* f. sp. *mustalae*; PC, *Pneumocystis carinii*; PW, *Pneumocystis wakefieldiae* (formerly *P. carinii* f. sp. *ratti*, also known as “variant”); PM, *P. murina* (formerly *P. carinii* f. sp. *muris*) PO, *P. carinii* f. sp. *oryctolagi*; CA, *Candida albicans*; SC, *Saccharomyces cerevisiae*. Genbank accession numbers were *P. carinii* (no. M25415), *Candida albicans* (no. J04230), and *Saccharomyces cerevisiae* (no. J02706). *P. carinii* f. sp. *oryctolagi* sequence was from [55,55a].

genes. Gene trees depict sequence divergence in two ways. The number of branches reflects the number of different sequences in the set used to make the tree. The degree of difference between sequences is depicted by the length of branches. Branch number and length combine to make a particular tree topology. The topology can be evaluated utilizing a test of reliability, such as the common bootstrap statistic [56]. To perform a bootstrap test, one sequence from the collection used to make a tree is randomly removed from the group and replaced with another from the same group (a procedure generally known as resampling with replacement). A new tree is then made from this altered data set. This procedure is repeated hundreds of times and the number of times a branch is present is tallied and expressed as a percentage. A bootstrap percentage value higher than 70% indicates that the branch in question appeared in more than 70% of trees. Such branches are generally considered to be strong—i.e., highly likely to reflect actual relationships, because they are not very dependent on the composition of the set of sequences being compared. In other words, the data set is sufficiently extensive to prevent distortions due to inadequate sampling.

Several recent studies of gene trees and their bootstrap values indicate that *Pneumocystis* organisms from different mammalian hosts contain distinct groups [33,57–61]. Most of these studies used a single locus to infer tree topologies. Table 3 lists the bootstrap statistics for tree branches derived from sequence comparisons involving three mitochondrial and four nuclear genes. Taken collectively, these data indicate that the *Pneumocystis* organisms (from human, monkey, mouse, rat, rabbit, and ferret) form a monophyletic group as compared to other fungi (bootstrap values range from 95 to 100% and average 98%). The table also shows that *Pneumocystis* organisms from rodents and primates are distinct from each other as well as from *Pneumocystis* organisms from ferret and rabbit fungi. (Bootstrap values for rodents vs. other species range from 96 to 100%, and average 98.6%; bootstrap values for primates vs. other species range from 57 to 100%, average 82.2%.)

Of course, the use of a gene tree for inferring organism relationships is subject to the caveat that a given gene might not be indicative of whole organism relationships, even when bootstrap values are high. The chance of encountering such an artifact is reduced by making trees for more than one gene. Making trees for multiple genes can also provide information about the number of biological species that contributed to the collection of genes being compared, because sexual reproduction can be detected [41].

Figure 4 shows trees for seven genes from *Pneumocystis* organisms from six different host species. The topologies of the trees are very similar, with rodents and primates each forming a group that is strongly supported with high

Table 3 Bootstrap Statistics from Published Comparisons of Sequences in *Pneumocystis* Species^a

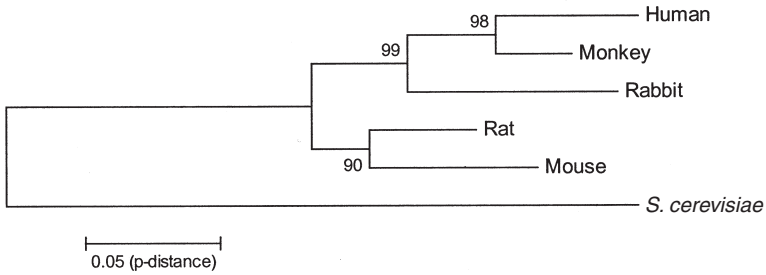
Sequence ^g	All <i>Pneumocystis</i> clades vs. other fungi	Rodent <i>Pneumocystis</i> vs. other <i>Pneumocystis</i>	Human vs. other <i>Pneumocystis</i>	Monkey vs. other <i>Pneumocystis</i>
SOD	100% ^a	100% ^a	100% ^a	100% ^a
DHPS	100% ^b	100% ^b	85% ^b	85% ^b
DHFR	100% ^b	96% ^b	57% ^b	57% ^b
TS	95% ^c	99% ^c	75% ^c	nb ^h
Cytb	99% ^d	nb ^h	nb ^h	nb ^h
mtrRNA(LSU)	nb ^h	97, ^e 100% ^f	77% ^f	77% ^f
mtrRNA(SSU)	nb ^h	98% ^f	99% ^f	99% ^f

Sources: References for Bootstrap statistic values:^a(57), ^b(60), ^c(59), ^d(61), ^e(58), ^f(33).

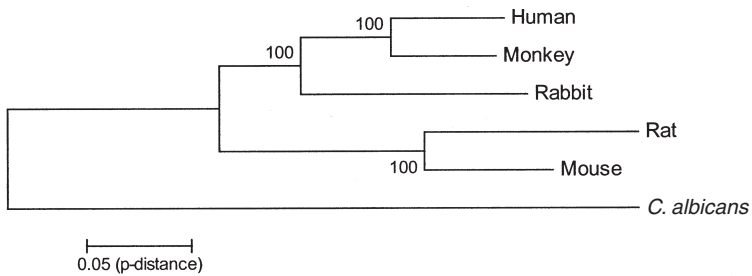
^g Loci analyzed encoded the following proteins and structural RNA species: manganese-cofactored superoxide dismutase gene (SOD), dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR), thymidylate synthase (TS), Cytochrome *b* (Cytb), mitochondrial large subunit ribosomal RNA (mtrRNA(LSU)), mitochondrial small subunit ribosomal RNA (mtrRNA(SSU)).

^h No bootstrap values were calculated.

(a)



(b)



(c)

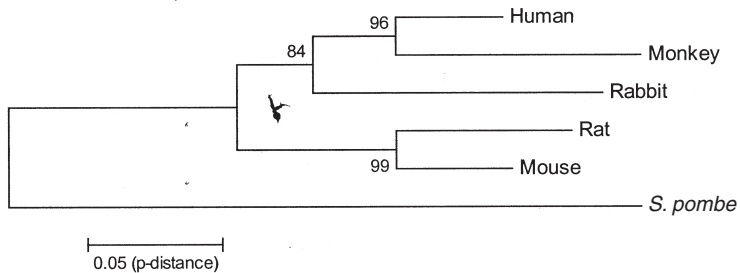


Figure 4 Phylogenetic trees of *Pneumocystis* sequences derived from six different mammalian hosts and seven genes. Two different monkey hosts were compared, owl monkey and macaque. Accession numbers used for these trees are given in Table 4. A. Portion of the mitochondrial small subunit ribosomal RNA gene. B. Portion of the manganese-cofactored superoxide dismutase gene. C. Portion of the mitochondrial large subunit ribosomal RNA gene.

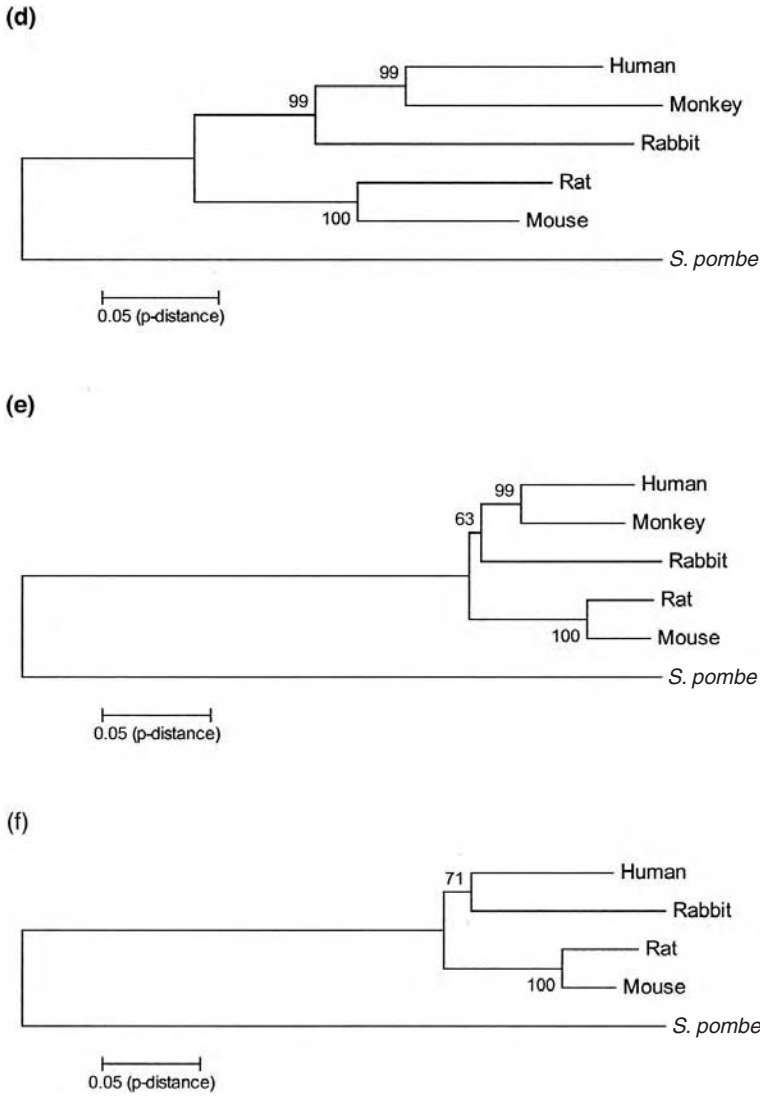


Figure 4 D. Portion of the dihydrofolate reductase. E. Portion of the dihydropteroate synthase gene. F. Portion of the enolpyruvylshikimate-3-phosphate synthase gene.

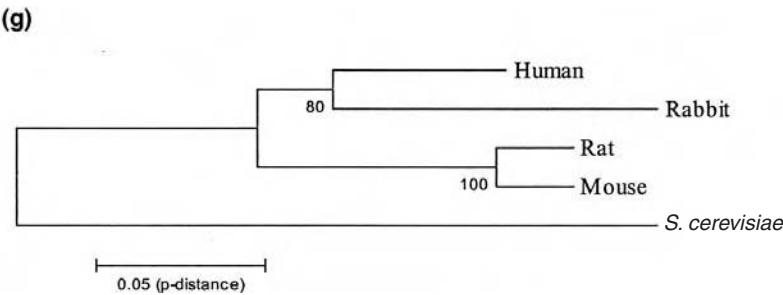


Figure 4 G. Portion of the thymidylate synthase gene (see Fig. 2 for nucleotide alignment). Genetic distances were calculated using p distance (i.e., the proportion of nucleotide sites at which the two sequences compared are different and is calculated by dividing the number of nucleotide differences by the total compared nucleotide sites). Neighbor-joining trees were inferred from nucleotide p-distance values using MEGA software, version 2.1 [66]. Near the nodes are bootstrap statistical percentages for 500 replications. Beneath the trees are the p-distance scales. Outgroup taxa: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans*.

bootstrap values, ranging from 90 to 100% and 96 to 100% for rodent and primate lineages, respectively. The bootstrap value for the rabbit branch is high as well (average bootstrap value is 85%). The branch lengths are all long, commensurate with the rRNA data described above.

The concordance of seven gene trees shows that the members of the genus are highly divergent throughout their genomes, which strongly supports the view that each of the six organisms is a separate evolutionary species. If this were

Table 4 Accession Numbers and References for Sequences Used to Construct Neighbor-Joining Trees in Figure 4

<i>Pneumocystis</i> from	Sequence						
	SOD	DHPS	DHFR	AROM	MtrRNA) (LSU)	MtrRNA (SSU)	TS
Mouse	AF146751	U66283	AF175561	U31053	AF257179	(58)	(59)
Rat	Z79785	M86602	AF322061	L18918	U20169	(58)	M25415
Human	AF146753	AF139132	AF090368	U31055	S42926	(58)	(59)
Monkey	AF146754	AY070270	AY017418	NA*	AF461783	(58)	NA
Rabbit	AF146752	AF322064	AF186097	U31054	S42915	(58)	(55)
Fungal outgroups	AF031478	AL031854	L13703	NC_003424	X54421	AJ011856	J02706
	C. albicans	S. Pombe	S. Pombe	S. Pombe	S. Pombe	S. cerevisiae	S. cerevisiae

Abbreviations: SOD, manganese-cofactored superoxide dismutase gene; DHPS, dihydripterolate synthase; DHFR, dihydrofolate reductase; AROM, enolpyruvylshikimate-3-phosphate synthase; mtrRNA (LSU), mitochondrial large subunit ribosomal RNA; mtrRNA (SSU), mitochondrial small subunit ribosomal RNA; TS, thymidylate synthase.
* No sequence available (NA).

not the case and *Pneumocystis* organisms were to reproduce via sexual means, the gene trees would tend to differ in topology, because sexual reproduction leads to random and independent assortment of alleles [41].

To summarize, all of the evidence, molecular genetic and functional, says that each host species has its own evolutionary species of *Pneumocystis*.

IV. Nomenclature

Scientific progress is promoted when a precise and simple system of nomenclature is used by all. Unfortunately, making nomenclature precise can be difficult due to the following dichotomy. On one hand, a name should indicate taxonomic rank. On the other hand, it is important to avoid the creation of unnecessary names. In other words, a species should have a species name, but a species name should not be assigned until species status is certain. Hence caution should rule, which has been the case in the management of *Pneumocystis* nomenclature, where there was no way to use mating experiments to directly test for biological species. However, a convincing case for multiple species has emerged from extensive genetic diversity measurements, and new names are needed to reflect this new knowledge.

To summarize, diversity in the genus *Pneumocystis* is much greater than it is among species in other fungal genera. Furthermore, the trees for seven genes are concordant, suggesting that the *Pneumocystis* organisms found in different host species do not mate. If they did, recombination would occur, causing discordance among gene trees. Finally, there is every indication that few *Pneumocystis* species share the same habitat, since they are host-restricted and do not proliferate much in culture, suggesting that they are obligate parasites. Thus, multiple factors prevent genes from moving between *Pneumocystis* species.

It seems clear that the genus is quite large and complex, containing many species. Therefore new names are required. The process of creating new species names has at last begun. Four of the best-studied species, those from humans, rats and mice, now have names that reflect their status. Obviously, there are many left to name. Later in this section, we summarize the steps entailed in assigning a new name. First, we must briefly review the history of *Pneumocystis* nomenclature and the names currently used.

The nomenclature applied to the genus has been discussed extensively during the last decade. Some proposed name changes appeared earlier [27,28], but it was not until 1990 that DNA sequence data forced reconsideration of the traditional practice of using one binomial, *P. carinii*, in reference to all types of *Pneumocystis*. As mentioned above, the inapplicability of the BSC and MSC engendered doubt when the possibility of multiple species was proposed. Most agreed, however, that it was desirable to have a nomenclature that would eliminate the need for verbose expressions like “*Pneumocystis* organisms derived from human.” In 1994, investigators attending the Third International Workshop on

Pneumocystis agreed to employ “special form” designations [62]. The following tripartite names were assigned: *Pneumocystis carinii* f. sp. *carinii* for the *Pneumocystis* found in most laboratory rats; *Pneumocystis carinii* f. sp. *ratti* for the second type of *Pneumocystis* found in laboratory rats; *Pneumocystis carinii* f. sp. *hominis* for human *Pneumocystis*; *Pneumocystis carinii* f. sp. *muris* for mouse *Pneumocystis*; *Pneumocystis carinii* f. sp. *mustelae* (ferret); *Pneumocystis carinii* f. sp. *equi* (horse); *Pneumocystis carinii* f. sp. *suis* (pig); and *Pneumocystis carinii* f. sp. *oryctolagi* (rabbit). Later, trinomial names were assigned to organisms isolated from shrews (*Sorex araneus*) *Pneumocystis carinii* f. sp. *sorex* [63] and different species of monkeys: *Pneumocystis carinii* f. sp. *callimico* (Goeldi’s monkey), *Pneumocystis carinii* f. sp. *callithrix* (Geoffroy’s marmoset), *Pneumocystis carinii* f. sp. *midas* (red-handed tamarin), *Pneumocystis carinii* f. sp. *fusci-colis* (Weddell’s tamarin), and *Pneumocystis carinii* f. sp. *sciureus* (squirrel monkey) [33].

As time went by, the data in favor of species became sufficiently convincing, and in 1999 the first new binomial names were published when Frenkel renamed a species found in rats *Pneumocystis carinii* Frenkel and a species found in humans *Pneumocystis jirovecii* Frenkel [49]. Other probable species in the genus are currently called special forms of *P. carinii* and will presumably continue to bear these names until new species names are assigned. The mechanism by which this should occur is defined by the International Code of Botanical Nomenclature (ICBN). The code is accessible on the Internet at <http://www.bgbm.fu-berlin.de/de/iapt/nomenclature/code/SaintLouis/0001ICSLContents.htm>.

The critical steps in the renaming process have been outlined [64]. Briefly, they are as follows: (1) Purified organisms should be deposited in a public collection that can be accessed by other investigators (Article 7, Recommendation 7A, Recommendation 8B). (2) The scientific name should follow Latin strictures (Principle V, Article 60). (3) Valid names are those that are validly published (Article 12). Valid publications require the following: (a) they must be published in a printed journal that is available to the general public, (b) the new taxon name must include both English and Latin descriptions (Article 36), and (c) scientific names of the hosts of parasitic fungi must be included in the publications (Article 32, Recommendation 32F). (4) When names are given by a single author, that author’s name should follow the new genus and species. For example, *Pneumocystis carinii* Frenkel [49]. If two authors were involved, their names should be separated by the word “et” or an ampersand (&). If more than two authors, the first author is followed by “et al” or simply “& al,” e.g. *Pneumocystis wakefieldiae* Cushion *et al.*

In addition to these ICBN requirements, we recommend the following: (1) Submit at least four sequences (e.g., three nuclear and one mitochondrial) and all other gene sequences to GenBank. (2) Include a phylogenetic analysis of the

sequences. (3) Submit DNA and any other genetic reagents such as DNA clone libraries to ATCC or a similar public repository.

V. Conclusion

It is obvious from Table 1 that recognizing species is not a simple matter. The long list of species concepts reflects the diversity of life forms that confront biologists. Each kind of organism has its own properties, and these both impose limits and present opportunities for investigation.

In the case of *Pneumocystis*, we have an abundance of gene sequence data. Phylogenetic analysis of these data clearly establishes variation at a level greater than that separating species in other fungal genera. Many investigators viewed these data at the Seventh International Workshops on Opportunistic Protists and indicated support for the view that new binomial names are both justified and needed. Obviously, we support this view.

The factor most likely to govern the rate at which new species of *Pneumocystis* are named is the pace at which the microbes that infect different mammals are subjected to sequence analysis. The picture presented in Figure 4 is clear. These five organisms are quite different at multiple loci. By contrast, the *Pneumocystis* organisms in most mammals have either not been analyzed at all or have been analyzed at just one locus.

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3

Pneumocystis Cellular Structure

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I. Introduction

The term *Pneumocystis carinii* names not only one taxonomic entity but a heterogeneous group of unicellular eukaryotic parasitic organisms included in the genus *Pneumocystis*, which has definitely been assigned to the fungal kingdom. Host species-related differences among *Pneumocystis* organisms from different mammalian species have been revealed at the genomic [1–8] and phenotypic levels, including ultrastructural morphology [9–12] or selective infectivity [9,13–16]. *Pneumocystis jiroveci* Frenkel 1999, formerly *P. carinii* f. sp. *hominis* [17,18], was the sole species identified in humans until recently. As viable isolates of *P. jiroveci* developed only limited growth in culture [16] and did not infect laboratory animals [16,19], most studies on *Pneumocystis* cellular biology were developed by using parasites of animal origin.

Most information about the cellular structure of *Pneumocystis* organisms was obtained thanks to improvements of transmission electron microscopy (TEM) techniques [20,21] as well as to the development of cytochemical methods [22–25] and three-dimensional (3D) reconstruction approaches adapted to *Pneumocystis* (see below). The high osmolarity of fixative and washing solutions revealed to be critical for obtaining well-preserved *Pneumocystis* cytoplasmic

structures for TEM. Furthermore, in vitro cultures and in vivo observations performed in highly reproducible animal models of *Pneumocystis carinii* pneumonia (PcP) [26] are producing new knowledge on the relationships of *Pneumocystis* organisms with epithelial alveolar cells or interaction with pulmonary surfactant. On the whole, this basic characterization of *Pneumocystis* spp. at the cellular level indicates that these organisms constitute a new biological group of atypical microscopic fungi that is opening a new field of basic biology research. This chapter updates the cell biology of *Pneumocystis* organisms, summarizing the present knowledge of their cellular structure, with data resulting from ultrastructural, 3D reconstruction and experimental studies developed in in vivo or in vitro models.

II. Morphological Methods for Studying *Pneumocystis* Organisms

A. Light Microscopy

Human-derived *Pneumocystis* organisms are usually detected in bronchoalveolar lavage fluid (BALF) or induced sputum samples by using light microscopy (Figs. 1 and 2) or immunofluorescence. In the first case, parasites, especially mature cysts, can be identified using phase contrast or Nomarski interference-contrast (Fig. 2). Most authors, however, currently detect these parasites on air-dried smears stained by toluidine blue O (TBO) [27], Gomori-Grocott's methenamine silver nitrate (GMS) [28], or methanol-Giemsa methods. TBO, cresyl violet [29], and GMS have a good affinity for components of the cyst wall (Fig. 1). Thus TBO stains the cell walls of cystic forms (i.e., intermediate and late sporocytes plus mature cysts) metachromatically in reddish violet. GMS stains this structure in dark brown. By using TEM, it was clearly shown that silver particles deposit on the glucan-rich electron-lucent middle layer of the cyst wall; in contrast, only little silver deposition was recorded in the electron-dense, unique layer of the thin trophic form's cell wall [24].

TBO or GMS facilitate rapid parasite detection, even at low magnification, in either clinical specimens or samples from experimental in vitro or in vivo models. However, these dyes also stain the cell wall of yeasts or other fungi. For this reason, in our experience, the best strategy to identify *Pneumocystis* organisms accurately is to systematically associate the examination of both TBO- (or GMS-) stained smears and methanol-Giemsa-stained smears from the same specimen. Actually, methanol-Giemsa (or other "panoptical" Giemsa-like stains) makes it possible, on the one hand, to distinguish *Pneumocystis* organisms from other microorganism and, on the other hand, to identify the different *Pneumocystis* life-cycle stages (Fig. 2). In fact, Giemsa and other stains with similar cytological affinities, such as Diff Quick [30] or RAL-555 [31–33], cause the parasite nuclei

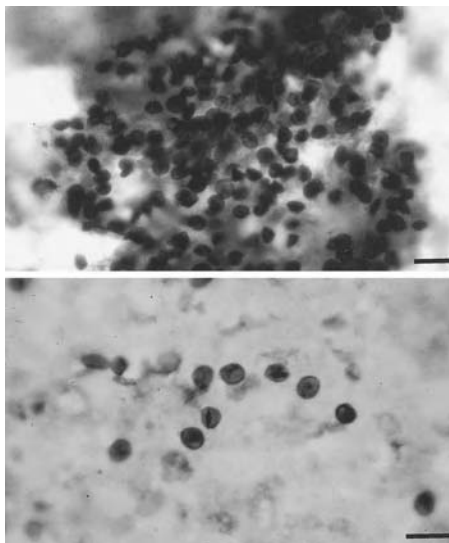


Figure 1 *Pneumocystis* cystic forms. Top: Human-derived parasites in a bronchoalveolar lavage sample stained by Gomori-Grocott's methenamine silver nitrate (GMS). Bottom: Rabbit-derived parasites stained by toluidine blue O (lung homogenate). Bar = 10 μ m.

to stain pinkish purple and the cytoplasm to stain blue. These polychrome stains make it possible accurately to distinguish *Pneumocystis* presumptive vegetative or cystic forms from other fungi and also from host cells or cell debris. Cystic or sporocytic walls are not stained by Giemsa or Giemsa-like methods, but they appear like a clear peripheral halo around cystic forms. Anyway, the biggest advantage of methanol-Giemsa or Giemsa-like stain methods consists in staining trophic forms, which remain unidentified in TBO- or GMS-stained smears [33]. To reveal *Pneumocystis* cystic forms in histological sections from lung or other organs, pathologists currently use these two last techniques. Furthermore, *Pneumocystis*-specific fluorescein- or peroxidase-labeled monoclonal antibodies provided by many suppliers may help to identify *Pneumocystis* organisms in touch preparations, lung-homogenate air-dried smears, or tissue sections.

Epon-embedded semithin sections have revealed themselves to be especially useful in *Pneumocystis* research studies. Current histological staining methods, such as periodic acid-Schiff (PAS), hematoxylin-eosin-saffron (HES), May-Grünwald Giemsa, or GMS were successfully adapted to stain high-resolution semithin sections from *Pneumocystis*-infected lungs (Fig. 2) [10,33]. Some of these protocols were speeded up by using a microwave oven.

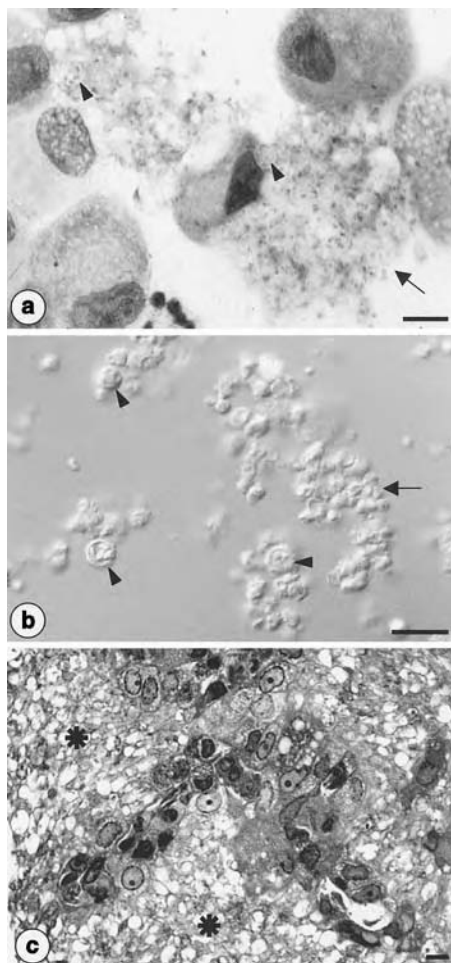


Figure 2 *Pneumocystis* organisms. A. Giemsa-stained cytopsin preparation of a human bronchoalveolar lavage sample. B. Rat-derived parasites observed in Nomarski interference-contrast. C. Pneumocystosis in a *Macaca mulatta* monkey (Indian subspecies). Lung semithin section stained by hematoxylin-eosin-saffron. Alveolar lumens (asterisks) are fully filled with *Pneumocystis* organisms. Alveolar septa are clearly visible. Arrows: clustered vegetative forms. Arrowheads: sporocytic or cystic forms. Bar = 10 μ m.

B. Transmission Electron Microscopy

Since the 1970s, many authors have developed TEM studies on *Pneumocystis* organisms. Unfortunately, the quality of fixation was often very poor, and these attempts (see Ref. 34 for review) provided only limited information. This situation led some authors to test different technical protocols so as to preserve the integrity of parasite cells [35–39]. Thus many fixative mixtures were tested, such as a phosphate-buffered glutaraldehyde-acrolein mixture [40], picric acid [40], a glutaraldehyde-acrolein-tannic acid mixture [35], a buffered potassium permanganate solution [36,40], a solution of glutaraldehyde-tannic acid in veronal or cacodylate buffer [35,41,42], ferrocyanide-osmium or tannic acid-osmium mixtures [20], uranyl acetate en bloc stain or phosphotungstic acid en bloc stain [42]. These efforts led to significant improvements of the quality of TEM preparations, especially of thick-walled forms (intermediate sporocyte to cyst stages) leading to significant progresses, which is examined in Section VI, below. However, as underlined by Ruffolo [34], no one fixation technique served to reveal all the ultrastructural features of *Pneumocystis* cells. What is more, diverse technical protocols did not protect the parasitic cytoplasm of vegetative forms from osmotic lysis [21]. Since the speed of penetration of fixing fluid is primarily affected by the fixative's tonicity [43], it was hypothesized that mixtures used in previous studies to preserve the integrity of *Pneumocystis* cells, which in fact lie beneath the pulmonary surfactant, were hypotonic. For this reason, the effect of a large range osmolarities of fixative or washing solutions (190 to 2580 mOsm) on the quality of fixation of samples from *Pneumocystis*-infected lung was tested [21]. Strikingly, high osmolarity (850 to 1300 mOsm) of fixative and washing solutions was revealed to be a critical condition for obtaining well preserved rabbit-, SCID mouse- or rat-derived *Pneumocystis* cytoplasmic structures for TEM (Fig. 3). Thanks to this improvement, a parasitic outer membrane—previously shown in cysts, spores, and young trophic forms [37–40,44]—was directly observed without difficulty in all the parasitic stages [21]. In addition, improved TEM studies, enriched with cytochemistry approaches, revealed in the *Pneumocystis* cell a complex endomembranous system as well as a large, active mitochondrion (see below).

In our experience [33], the osmolarity can be decreased to 650 to 750 mOsm; in these conditions, the rate of well-preserved vegetative forms is lower, but lung tissues are less affected by hyperosmolarity. What is important is that after the fixation step, washing must be performed in buffer solutions with a tonicity identical to that of the fixative solution. Thus, a good protocol to fix *Pneumocystis* parasitized lung samples from humans or laboratory animals is the following: (1) fixation with a phosphate-buffered 2.5% glutaraldehyde solution (0.1M pH 7.5) adjusted to about 700 mOsm by the addition of 0.18M NaCl; (2) many washings with 0.1M phosphate buffer (same osmolarity); [3] postfixation

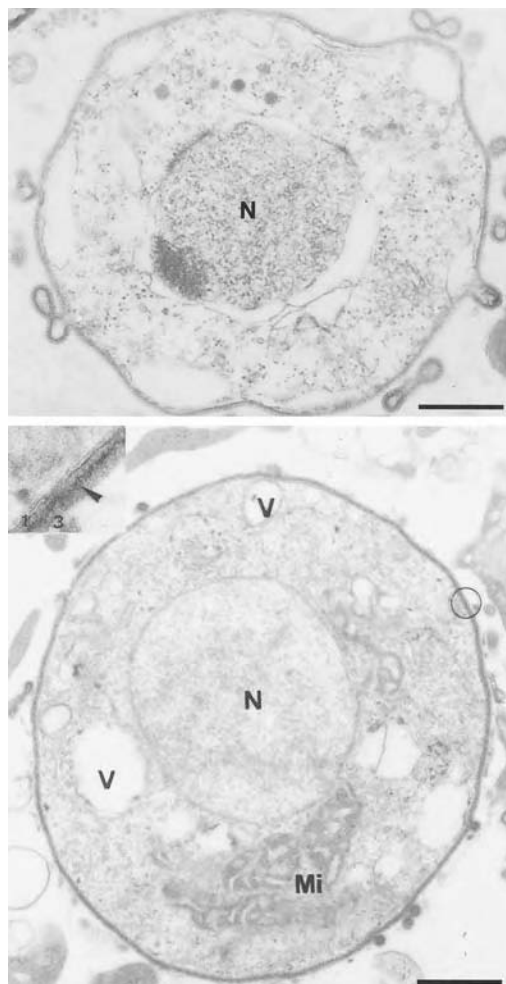


Figure 3 Rabbit-derived *Pneumocystis* organisms prepared for transmission electron microscopy (TEM) in different conditions. Top: Large trophic form fixed in isosmotic solution. Osmotic lysis and cytosolic extraction are visible. Only a few osmiophilic granules can be recognized in the cytoplasm. Bottom: Early sporocyte fixed and washed in hyperosmotic solution (850 mOsm) in which mitochondrial cristae (Mi), vacuoles (V), and other cytoplasmic structures are clearly visible. The outer membrane (arrowhead in the inset) appears as an electron-dense deposit in the outer electron-dense layer of the cell wall (circle and inset). N = nucleus. Bar = 0.25 μm .

for 1 hr in a 1% osmium tetroxide solution in phosphate buffer, dehydration in ethanol, and embedding in Epon [33].

C. Immunoelectron Microscopy (IEM)

Hyperosmolar conditions are also needed to obtain well-preserved *Pneumocystis* samples for immunocytochemistry studies [22,45]. Molecular sites reacting specifically with lectins or antibodies as well as the fine structure of the parasite were well preserved in *Pneumocystis* samples embedded in hydrophilic resin (London resin white) [22]. Surface antigens were immunodetected at the ultra-structural level in different life-cycle stages of the parasite by using monoclonal or polyclonal antibodies secondarily revealed by colloidal gold particle-conjugated antibodies [22]. Antisera were also used to localize *Pneumocystis* cell components. For example, beta-1, 3-glucan was immunolocalized in *Pneumocystis*-infected rat lung tissue by using immunogold labeling. A high concentration of beta-1,3-glucan was found in the electron-lucent layer of the parasite's cyst wall but was apparently absent from the cell wall of spore or trophic forms [46]. In other works, monoclonal antibodies revealed antigenic differences in terms of the host species [47]. One antibody reacted with the outer membrane of human-derived *Pneumocystis* organisms but not with the rat-derived *Pneumocystis* homologous structure; another recognized selectively the cystic form of both human and rat parasites. Furthermore, gold-labeled antibodies were also used to reveal the cytoskeletal proteins of rat-derived *Pneumocystis* [48]. Actin was found to be irregularly scattered in the trophic form's cytoplasm and more concentrated in the inner electron-lucent layer of the cystic cell wall, whereas tubulin was localized at the trophic form's peripheral cytoplasm and along filopodia.

III. Life Cycle of *Pneumocystis* Organisms

All known *Pneumocystis* life-cycle stages were found in the lungs of infected hosts, though exceptionally parasites may spread to other organs. On the basis of TEM data (Fig. 4), it is usually accepted that the life cycle of *Pneumocystis* organisms involves a thin-walled mononuclear presumptive vegetative or trophic form, which becomes a thick-walled cystic stage (cyst or spore case) in which a multiple nuclear divisions lead to the formation of eight spores [41,49]. These forms could likely leave the cyst, presumably by a preformed pore located at the thickest part of the mature cyst's cell wall [50], to attach specifically to type I epithelial alveolar cells and evolve into the cystic stage. Most authors believe that trophic forms are able to multiply by binary fission. This point is examined in detail in Section IV.

The transition from trophic form to mature cyst (containing eight spores) seems to occur through three consecutive sporocytic stages [41,51,52] (Fig. 4):

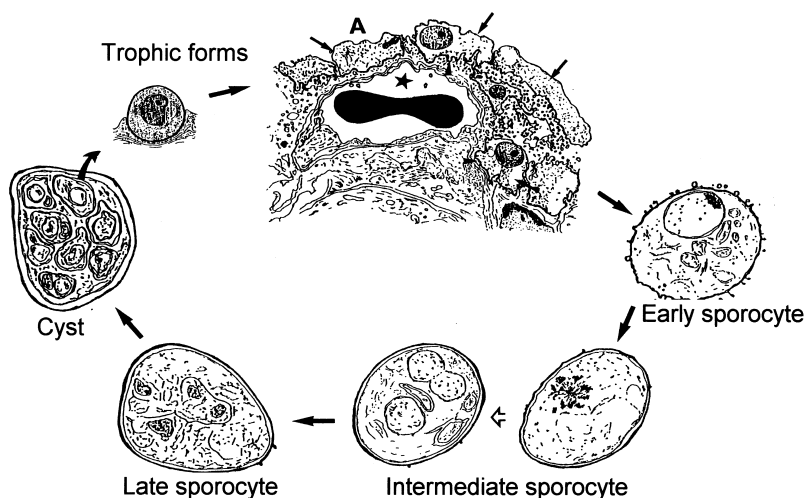


Figure 4 A hypothetical life cycle of *Pneumocystis* spp. Parasites are represented as observed in the lung using TEM. Pleomorphic, thin-walled mononuclear trophic forms are shown attached to type 1 epithelial alveolar cells close to an alveolar capillary vessel (star). Trophic forms (small arrows) evolve into thick-walled sporocytic and cystic stages, in which a multiple nuclear division leads to the formation of eight spores. These forms are able to leave the cyst and to attach specifically to type 1 epithelial alveolar cells. (Modified from Ref. 135.)

(1) a spheroid, large, mononuclear early sporocyte with a thin electron-dense cell wall similar to that of trophic forms. Synaptonemal complexes were observed in the nucleus of this stage, indicating the occurrence of a meiotic division—i.e., of a sexual cycle [41]; (2) a multinuclear (from two to eight nuclei) intermediate sporocyte with a thick, two-layered cell wall similar to that of the cystic stage—i.e., with an electron-lucent layer inside the electron-dense layer found at other stages (Fig. 4); (3) an eight-nuclear thick-walled late sporocyte containing recognizable spores that are not yet fully delimited. A schematic characterization of each *Pneumocystis* life-cycle stage is attempted in Table 1.

The formation of the spore results from invaginations of the late sporocytic cell membrane. Each mononuclear spore presents a unique, dense mitochondrion, a well-developed rough or smooth endoplasmic reticulum, and an electron-dense one-layered cell wall, which is externally lined by the outer cell membrane [21,37–40] (see Sec. VI).

Until now, the diverse stages of *Pneumocystis* species were named by using a terminology that evokes the terms usually applied to protozoan organisms. At present, taking in consideration the close phylogenetic relationships between

Table 1 Life Cycle Parasitic Stages of *Pneumocystis* Organisms

	Trophic Form	Early sporocyte	Intermediate sporocyte	Late sporocyte	Cyst
Light microscopy					
<i>TBO</i>	–	–	+	+	+
<i>Giemsa</i>	+	+	+	+	+
Transmission electron microscopy					
<i>Shape</i>	Irregular	Ovoid	Ovoid	Ovoid	Ovoid
<i>Diameter (μm)</i>	2–8	3.5–4.5	4–6	4–6	4–7
<i>Number of nuclei</i>	1	1	2–8	8	8 ^a
<i>Synaptonemal complexes</i>	No	Yes	No	No	No
<i>NAO^b</i>	No	No	Yes	Yes	No
<i>Nuclear spindle</i>	No	Yes	Yes	No	No
<i>Mitochondrion</i>	Elongated, little ramified	Ramified	Dendritic	Split	Ovoid, dense
<i>Cell wall (diameter in nm)</i>	Electron-dense layer (20–25 nm)	Electron-dense layer (35–45 nm)	Electron-dense + inner electron-lucent layers (80–100 nm)	Electron-dense + inner electron-lucent layers (80–120 nm)	Electron-dense + electron-lucent layers (80–120 nm)
<i>Filopodia</i>	Numerous	Poorly developed	Scarce	Scarce	Scarce

Key: +, stained; –, unstained; TBO, toluidine blue.

^a One nucleus in each of the eight spores.

^b Nuclear-associated organelles.

Pneumocystis and Ascomycetes [6,53–55], it was proposed to rename the *Pneumocystis* stages by using terms usually applied to fungal development stages [34]. In this way, the cyst should be named *ascus*. Consequently, as most asci contain eight ascospores at maturity, this term could be employed for naming the eight spores contained in the *Pneumocystis* “cyst”.

However, as reviewed in Sections IV and V, efforts are still needed to determine accurately the ploidy of different *Pneumocystis* stages as well as the occurrence of conjugation and other typical events of ascomycetous developmental biology. Furthermore, it should be remembered that at least one extensive phylogenetic study has shown a close relationship between *Pneumocystis* species and usteomycetous fungi [1], a finding that is consistent with some biochemical features shared by *Pneumocystis* organisms and basidiomycetous rust fungi (see Sec. VI.A). For these reasons, in the present chapter, *Pneumocystis* life-cycle stages were named by using terms that do not specifically target a defined fungal group—i.e., that may be applied to fungal organisms as a whole [34].

IV. Cell Division in *Pneumocystis*

Micrographs of *Pneumocystis* nuclear divisions (Fig. 5) are rare [34]. At our present state of knowledge and in our own experience, typical microtubular spindles were adequately documented only in *Pneumocystis* early sporocyte or other sporocytic stages [51,52]. Although binary fission of *Pneumocystis* sp. trophic forms was suggested on the basis of TEM micrographs, interpretation of the available published images remains problematic. The main problems derive from the following facts: the highly irregular, pleomorphic shape of trophic forms, the uneasy distinction between early sporocytes and trophic forms, and the uneasy discrimination between potential binary fission and conjugation. The irregular shape of trophic forms prevents the observer from considering that cytoplasm or even nuclear constrictions observed in ultrathin sections of trophic forms are necessarily indicative of binary fission. The presence of nuclear changes usually associated with mitosis might make it possible to avoid this difficulty. However, how can it be shown that the observed cell is rather a “rounded” trophic form than an early sporocyte (where nuclear division leads to mature cyst and not to binary fission)? This may be the case in one of the more suggestive published pictures of the vegetative form’s division (see Fig. 20 in the Ref. 51). Last, when one sees an apparent vegetative form with two nuclei, as nicely shown by Itatani [56], how is one to distinguish binary fission from conjugation? This difficulty was mentioned by Yoshida [51] and well discussed by Itatani [56]. 3D reconstruction from thin sections (both for facilitating the distinction between the trophic form and the early sporocyte and distinguishing cytoplasmic or nuclear constrictions from real binary fission), plus high-osmolarity fixation (for properly fixing

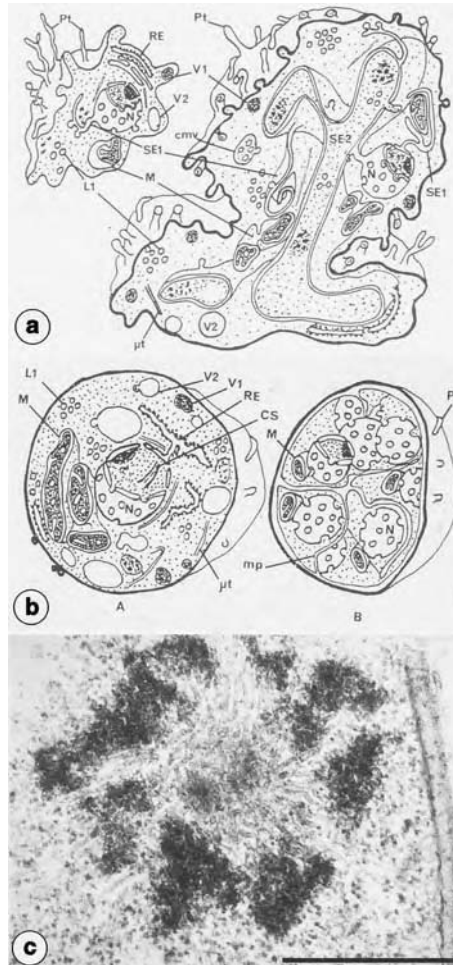


Figure 5 Cellular structure of rabbit-derived *Pneumocystis* organisms. A. Three-dimensional representation of small and large trophic forms. B. Early sporocyte (left) and late sporocyte (right). cmv = multivesicular body; CS = synaptonemal complex; M: mitochondria; mp = plasma membrane; N = nucleus; μ t = microtubules; RE = endoplasmic reticulum; Pt = filopodia; SE1, SE2 = type 1 or 2 endoplasmic saccules; L1 = primary lysosome; V1 = osmiophilic granule; V2 = vacuole. (Modified from Ref. 136.) C. Nuclear division in an intermediate sporocyte. Condensed chromosomes and spindle microtubules are clearly visible. Bar = 0.1 μ m.

the nuclear envelope and associated structures) should be helpful in elucidating the outcome of the *Pneumocystis* trophic form.

From another point of view, the absence of distinct evidence of trophic form division could result from the low frequency of this process, the high speed of division, or the actual absence of trophic form binary fission. A low frequency of parasite multiplication seems to contradict the fact that trophic forms usually represent more than 95% of parasites found in infected lungs. Moreover, current photonic or electron microscopy observations indicate that parasite proliferation is asynchronous—i.e., at a given moment, diverse parasitic stages coexist either in the pulmonary alveolus or in culture. Therefore if binary fission occurs, it should frequently be observed.

If trophic forms do not divide, the high number of parasites observed in the lungs of susceptible hosts should result exclusively from cyst development. In order to explore this important point, the contribution of each parasite stage of *Pneumocystis* growth was investigated in vitro in both feeder cell and axenic short-term cultures of rat-derived parasites [57]. Kinetics of each stage were studied to ascertain that growth ensues from either multiplication of the trophic form cyst formation, or both. In either feeder cell or axenic cultures, an increase of the number of filled (i.e., containing spores) or empty cysts was recorded, attesting that cysts contributed to parasite growth. Indeed, the increased population of empty cysts indicated that trophic forms were produced from filled cysts. Likewise, the increased number of filled cysts indicated that trophic forms evolved into filled cysts. It was therefore concluded that, in the two explored systems, trophic forms resulted from cyst development [57]. Using *P. carinii*–A549 epithelial alveolar cell cocultures, Cushion et al. [58] found that excystment could take place in vitro, but they conclude, on the basis of differential parasite stage counts, that growth resulted essentially from trophic form binary fission. However, whereas the results of Cushion et al. [58] were essentially based on parasite counts in culture supernatants, Aliouat et al. [57] enumerated only the parasites that attached to host cells. This methodological difference prevents any comparison of results.

The next question was as follows: can trophic forms result also from trophic form division? It was hypothesized that if trophic forms do not divide, they must all result from cyst evolution. In fact, each time a filled, mature cyst becomes an empty cyst, eight trophic forms are released. In this case, the empty cyst vs. trophic form growth-kinetic curve should be a straight line, with a slope equal to 8. In cultures on L2 lung epithelium-like cells from rat origin (ATCC N° CCL 149), the slope was actually 8.08, suggesting that one trophic form produces only eight trophic forms by cyst production. In axenic conditions, the slope was 10.2, suggesting that trophic forms result mostly from cyst development but that some types of trophic form division could also contribute to the parasite's growth [57].

The hypothesis that *Pneumocystis* growth results essentially or perhaps exclusively from cyst development and not from trophic form binary division was further supported by the response of *Pneumocystis* infection to antifungal echinocandins, inhibitors of beta-1,3 glucan synthesis, or sordarins, inhibitors of fungal protein synthesis. Actually, an echinocandin (L-671,329) was able to selectively eliminate *Pneumocystis* cysts in infected rats submitted to therapeutic doses of the drug. Then, using lower, prophylactic doses of the same molecule, Schmatz et al. [59] prevented the appearance of trophic forms. As the drug did not directly affect these forms, the authors suggest that the cyst stage was required for trophic form production. Similarly, some sordarin derivatives, which were revealed to be highly active anti-*Pneumocystis* drugs [60], seemed to have a preferential effect against cystic forms in experimental *Pneumocystis* pneumonia (PcP) in rats [61]. Indeed, the problem of the contribution of different life-cycle parasite stages to the parasite's growth needs to be further explored; it is both a key problem of *Pneumocystis* basic biology and an essential question to understand the mechanism of action of anti-*Pneumocystis* drugs.

V. *Pneumocystis* Ploidy

The occurrence of meiosis in the mononuclear early sporocyte was attested by the detection of synaptonemal complexes that conduct the alignment of homologous chromosomes at the prophase of the first meiotic division [41]. The second meiotic division should produce four haplophasic nuclei. Then a final mitotic division should lead to the eight nuclei observed in the late sporocyte and in the mature cyst (Fig. 4). If this view is right, the unique nucleus of the spore would be haploid, and a process of conjugation (or some kind of nuclear fusion) should occur in order to produce diplophasic forms able to restart the mature cyst generation process.

Although these hypotheses were little explored, some recent observations have proved to be consistent with them. First, ultrastructural evidences of a process similar to *S. cerevisiae* conjugation were recently reported in rat-derived *Pneumocystis* organisms [56]. Second, haploid and diploid conditions seemed to coexist in *Pneumocystis* populations, as was shown by using the fluorescent double-stranded nucleic acid 4',6-diamino-2 phenylindole (DAPI) stain [62], other fluorescent stains, or Feulgen cytophotometry [63], though most organisms were haploid.

In contrast, when 15 cloned rat-derived *P. carinii* (formerly named *P. carinii* f.sp. *carinii*) sequences were mapped to specific bands in electrophoretic karyotypes, the results suggested that the organism was haploid [64,65]. However, pulsed-field gel electrophoresis (PFGE) was performed from a heterogeneous population of vegetative, sporocytic, and cystic forms extracted from rat lungs, where more than 90% are usually presumptive vegetative forms (trophic forms).

Or, if conjugation is a relatively infrequent event [56], the very low proportion of diplophasic forms could explain why no evidence of diploidy was observed in such experiments. Consistently, a karyotype and restriction display (KARD) two-dimensional pulsed-field electrophoretic analysis of the *P. carinii* genome revealed 17 chromosomes and the presence of homologues for at least two chromosomes—an observation that supports the hypothesis of a diploid condition for the *Pneumocystis* nucleus [66].

VI. General Ultrastructure of *Pneumocystis* Organisms

For a long time it was held that *Pneumocystis* organisms had indistinct nuclei, scarce cytoplasmic organelles, poorly developed mitochondria, and a poorly organized endoplasmic reticulum. This wretched image of the parasite, which resulted from the fact that up to 1989–1990 neither a Golgi complex nor a lysosomal system was known in any *Pneumocystis* stage, was rectified thanks to improvements in methods of preparing *Pneumocystis* samples for TEM [20–22]. At the same time, cytochemistry, serial sectioning and 3D reconstruction methods were applied to the ultrastructural study of the parasite. These approaches revealed a highly complex endomembranous system in *Pneumocystis* that includes rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER), Golgi vesicles, lysosomes, and complex endoplasmic saccular structures [67–70]. In this section, the cell structure of each *Pneumocystis* stage is examined.

A. Trophic Form and Early Sporocyte

Trophic forms and early sporocytes are the presumptive vegetative life-cycle stages of *Pneumocystis*, having a thin cell wall that consists of only one electron-dense layer. Trophic forms usually have a complex, irregular, or “amoeboid” shape. However, the smallest forms (Figs. 4 and 6) are round to ellipsoid, appearing as 1- to 2- μm eukaryotic cells. These are probably the youngest organisms, newly released from the cyst. Their cytoplasm contains one nucleus (up to 1 μm in diameter) that has a typical nuclear envelope with clearly visible nuclear pores. The chromatin generally appears diffuse. The perinuclear cisterna communicates with well-developed RER or SER [67]. A low number of Golgi vesicles (less than 20 as shown by ultrastructural 3D reconstruction) and only one mitochondrion, somewhat irregular in shape, are present [68]. The cytoplasm contains 50- to 70-nm osmiophilic granules that are probably lipoid in nature. Filopodia are rather poorly developed in this small cell, which closely resembles the spores described below.

Most trophic forms are larger and very irregular in size (4 to 8 μm long) and shape (Fig. 5). When high-osmolarity fixative has been used (see Sec. II.B), trophic forms can easily be recognized on the basis of their electron-dense cell

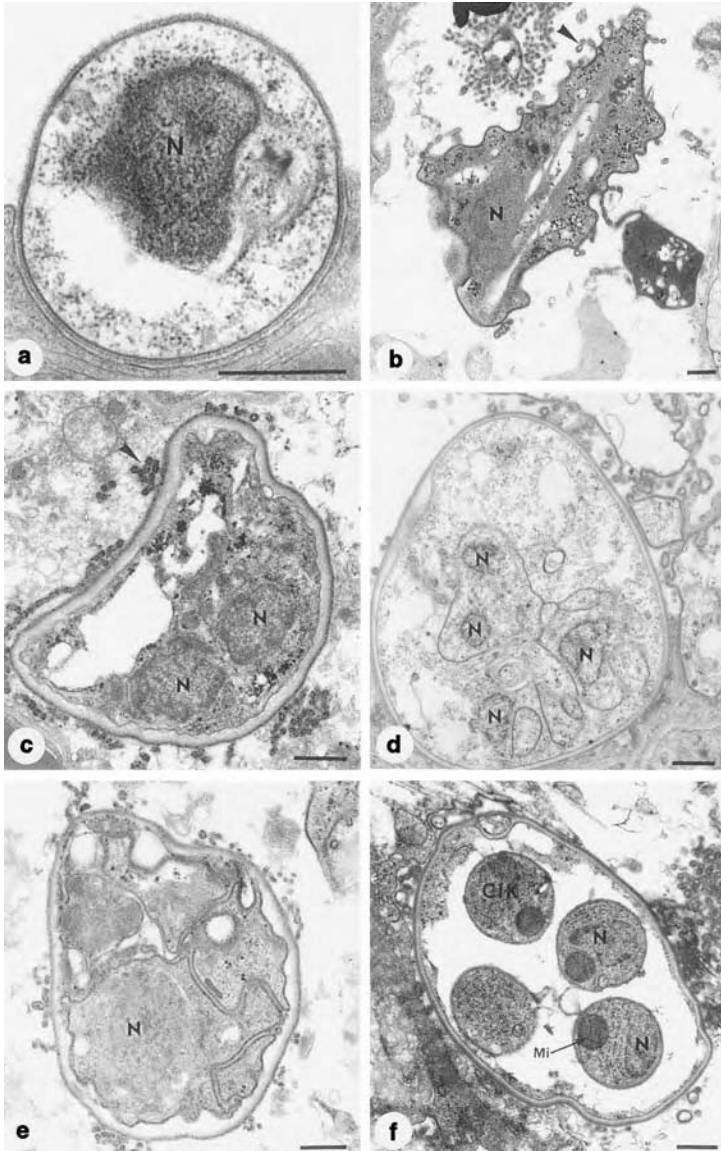


Figure 6 Life-cycle stages of *Pneumocystis* in TEM. A. Young trophic form attached to a type 1 epithelial alveolar cell. Host: rabbit. B. Pleomorphic trophic form in the alveolar lumen. Host: rat. C. intermediate sporocyte: the typical cell wall and two nuclei are well visible. Host: rat. D. Another intermediate sporocyte where the formation of spores is more advanced. Host: rabbit. E. Mature cyst containing “amoeboid” spores. Host: rat. F. mature cyst with spheric spores. Host: rat. CIK = spores; Mi = mitochondria; N = nucleus. Arrowheads = filopodia. Bar = 0.5 (From Ref. 137.)

wall, their relatively dense appearance, and their great number of filopodia. The cell wall, 20 to 25 nm thick, consists of an electron-dense layer associated with the outer face of the plasma membrane (6 to 7 nm), which extends from the cellular body to the filopodia. Therefore the surfaces of these structures—which are frequently observed in cross, oblique, or longitudinal sections—constantly show cell wall, plasma membrane, and cytoplasm levels. Filopodia are clearly visible in the scanning electron microscope (SEM) (Fig. 7). They are filiform, tubular expansions with either a regular diameter or localized swellings. In mouse-

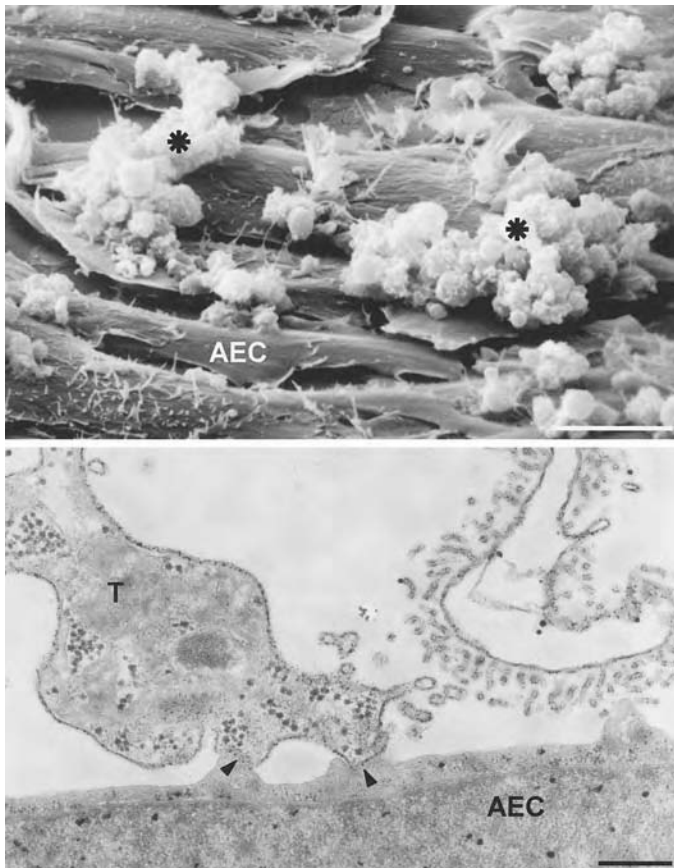


Figure 7 In vitro rat-derived *Pneumocystis* cocultured with L2-rat epithelial alveolar cells (AEC). Clustered parasites (asterisk) are clearly visible in scanning electron microscopy (top). Bar = 10 μm . Attachment (arrowheads) of a trophic form to an epithelial cell observed in TEM (bottom). Bar = 0.5 μm

derived *Pneumocystis*, filopodia are markedly abundant and thin; they develop tree-like branching [11].

The cell wall of both early sporocyte and trophic forms has an outer membrane that appears as a more or less discontinuous electron-dense osmiophilic deposit that lines the trophic form's plasma membrane or is embedded in the early sporocyte's cell wall (Fig. 3). As indicated in Section IV, the outer membrane is already present in the spore. After excystation, when the spore evolves into a trophic form and this in early sporocyte, the outer membrane is observed in the electron-dense layer of the cell wall. In thick-walled *Pneumocystis* stages, the outer membrane appears as an osmiophilic, discontinuous structure situated in the electron-dense outer layer of the cell wall close to the inner electron-lucent layer. The *Pneumocystis* outer membrane, which is rather like that of gram-negative prokaryotes and blue-green algae, appears as a peculiar structure that is apparently absent from the cell wall of other fungi. Its biological function remains unknown. It could be involved in osmotic regulation, selective molecular transport through the cell wall, or other functions [21,44].

Plasmalemma or endoplasmic membranes of rat-derived *Pneumocystis* organisms were studied by using freeze-fracture methods. Intramembranous proteins (IMP) were found to be more numerous on the P face (protoplasmic half-membrane, more than 1000 IMP/ μm^2) than on the E face (exoplasmic half-membrane, about 400 IMP/ μm^2) of the trophic form plasma membrane [71–73]. This was verified in every *Pneumocystis* stage. However, total IMP density decreased progressively as the parasite proceeded in its development from trophic form into cyst, suggesting that presumptive vegetative forms are metabolically more active than cystic forms.

Cholesterol, which constitutes about 78% of total sterol in rat-derived *Pneumocystis* [74], is largely present in the parasitic plasma membrane, as shown by using filipin, a polyene antibiotic with high affinity to 3-beta-hydroxysterols [75]. By using freeze-fracture, filipin-sterol complexes were found to be more numerous on the P face than on the E face of *Pneumocystis* plasma or endoplasmic membranes, and their number decreased through the development from trophic form to cyst stage [75]. Interestingly, *Pneumocystis* spp., like rust fungi, do not have ergosterol and have *cis*-9,10-epoxyoctadecanoic acid, a rare fatty acid that occurs in high concentration in the uredospores of rust fungi [76]. The absence of ergosterol can explain why *Pneumocystis* organisms were not susceptible in vivo to amphotericin B, another antifungal polyene, though in vitro this drug apparently binds to *Pneumocystis* sterols and causes permeability changes in the parasite membranes [77].

Trophic form or early sporocytes have a single nucleus, which occupies more than 30% of the cell volume of trophic forms [68] and is surrounded by a nuclear envelope that exhibits numerous nuclear pores (eight pores per square micron) [51] (Fig. 5). Nuclear pores, mitochondria, and other cellular structures

of the rabbit-derived *Pneumocystis* organisms [67] were comprehensively studied by using zinc iodide–osmium tetroxide (ZIO), a reagent that enhances the endomembranous system of eukaryotic cells [78]. This approach revealed that nuclear pores have a diameter of 55 to 80 nm. As in small trophic forms, the perinuclear cisterna communicates with RER or SER saccules. The presence of synaptonemal complexes in the nucleus of the early sporocyte and its biological significance were analyzed in Sections III to V above. A nucleolus is often visible in the trophic form (Figs. 5 and 7).

The endomembranous system of rabbit-derived *Pneumocystis* showed two types of endoplasmic structures closely related to RER, SER, and the Golgi complex (Fig. 5). The first, which was named a type 1 endoplasmic saccule (ES₁) [67], consisted of one or more coiled endoplasmic saccules that packaged cytoplasm or mitochondria, suggesting autophagic activity. ES₁ could therefore be considered secondary lysosomes. The second type, which was named a type 2 endoplasmic saccule (ES₂), consisted of a large, flattened, single endoplasmic saccule present in well-developed trophic forms and in intermediate sporocytes. Although it seems to appear just before nuclear division, its function remains unknown.

Vesicles of a golgian nature, as revealed by the cytochemical demonstration of thiamine pyrophosphatase (TPPase) activity [67], which is a marker of the *trans* face of the Golgi complex [79], were often observed next to ES₁ or ES₂ (Fig. 5). Cytidine 5′monophosphatase and beta-glycerophosphatase activities were also demonstrated in these vesicles [67], which developed by budding from endoplasmic membranes as well as from the nuclear envelope [80]. Their number increased as parasites proceeded in their development from the trophic form to the intermediate sporocytic stage. This is probably due to the fact that the transition from monolayered forms (trophic form and early sporocyte with a cell wall consisting of only an electron-dense layer) to bilayered forms (intermediate, late sporocyte, and cyst, with a cell wall consisting of an external electron-dense layer and an internal electron-lucent one) is associated with an increased synthesis of cell wall compounds. 3D reconstruction studies (Fig. 5) have shown that large trophic forms and intermediate sporocytes displayed more than 200 Golgi vesicles [68,69].

Computer-aided 3D reconstruction from serial thin sections [68] has revealed that trophic forms of rabbit-derived *Pneumocystis* have a single mitochondrion with budding zones that occupies an important volume in the cell (Fig. 5). Serial sectioning and cytochemical methods made it possible to establish that rabbit-derived *Pneumocystis* mitochondrial cristae present a broad base and an upper rather tubular portion (unpublished observations).

Trophic forms, like other *Pneumocystis* sp. life-cycle stages, present a variety of other structures, such as osmiophilic granules, vacuolar spaces, and microtubules [21,42,80–82]. In contrast, neither flagella nor cilia were observed in any *Pneumocystis* sp. life-cycle stage.

B. Intermediate and Late Sporocytic Stages

Sporocytes were briefly characterized in Section III and Table 1. The key event of the early to intermediate sporocyte transition is the development of the bilayered cell wall that will remain practically unchanged in late sporocytes and mature cysts (Fig. 6, Table 1). The monolayered cell wall of the early sporocyte becomes thickened by the appearance of a new electron-lucent middle layer. Thus, development from the early to the late sporocyte is associated with an increase in cell wall thickness from 40 to 100 nm [51,52,80]. The outer membrane is placed on the electron-dense layer very close to the electron-lucent layer [21,36,80].

Carbohydrate was detected in the *Pneumocystis* sporocyte or cyst wall at light microscopy level by periodic acid–Schiff (PAS) staining [25] and at the ultrastructural level by Thiéry staining, which stained only the electron-lucent layer [25,67]. In the same way, the cell wall of *Pneumocystis* cystic organisms was reactive to the lectin concanavalin A (ConA) [25,67,83,84]. The results of the analysis of carbohydrates of the *Pneumocystis* cyst wall showed that the total sugar composition was on the order of 75 to 80 mg/mg dry weight (8%), which is a low amount in relation to that of most fungi [85]. In the cyst wall, glucose was found to be the major sugar constituent. Mannose and galactose were present in equal proportion, and a lesser amount of *N*-acetyl-D-glucosamine, as well as trace amounts of ribose and sialic acid, which were detected in the same kinds of samples [85]. The presence of beta-1,3 glucan in the cyst wall of *Pneumocystis* was suggested on the basis of stain affinities and enzymatic assays [85–87]. Specific beta-1,3-glucan antibody localized glucan to the cystic wall [46], and the use of specific beta-1,3-glucanases has made it possible to detect glucan in the electron-lucent layer of the cell wall [87]. The cystic wall also has chitins [88].

In the cell wall of all parasite stages there is a 116- to 120-kDa mannose-rich glycoprotein that represents the major surface antigen of *Pneumocystis*. This highly polymorphic glycoprotein—also called gpA, major surface antigen, or major surface glycoprotein (Msg)—is encoded by a family of about approximately 100 different genes, which are organized in clusters located at the ends of each of the chromosomes [65]. Msg binds specifically to surfactant protein-A (SP-A) [89,90] and to fibronectin [91]. The quantitative importance of *Pneumocystis* Msg molecules, their immunogenic power, as well as their biological properties suggest that they play an important role in the ability of *Pneumocystis* to colonize the lung alveolar spaces and escape via unspecific or specific defense mechanisms. Interestingly, there is a connection between the MSG protein family and a polymorphic *Pneumocystis* subtilisin-like serine protease (Prt 1) [92], which is also encoded by a multigene family. Genes of the PRT family tend to be linked to genes of the MSG family, and the production of Msg may involve a proteolytic cleavage of a pre-Msg by subtilisin-like serine proteases [65].

In early and intermediate sporocytes, the mitochondrion becomes highly complex [68,69]. This organelle is unique in the parasite cell until the formation of the spores in intermediate and late sporocytes. The mitochondrion, which is ovoid in spores and in small trophic forms, is larger in the other stages, where it develops budding. Thus, in the intermediate sporocyte, the mitochondrion occupies a large portion of the cytoplasm and develops active budding, becoming spiral and somewhat tree-like [69]. Probably budding processes observed in trophozoitic or sporocytic mitochondria will evolve into individual mitochondria in spores. These parasitic stages are already recognizable in the late sporocyte. Actually, in this stage, the plasma membrane develops internal budding to envelope portions of cytoplasm that will evolve into well-individualized spores, each with a single nucleus, a single mitochondrion, and abundant RER (Fig. 6).

C. Cystic Stages and Spore

Mature cysts of *Pneumocystis* spp. are about 4 to 7 μm in diameter, round, and thick-walled (Fig. 6). Its surface is rather smooth, with rare filopodia. When the membrane around each nucleus and its adjacent cytoplasm are formed, a maximum of eight spores is found in the cyst.

The cyst wall has features roughly similar to those of the cell wall of intermediate and late sporocytes. However, in the mature cyst the bilayered cell wall presents a thickened portion that protrudes inward, ranging from 1 to 2 μm in width and 200 to 300 nm in thickness [51]. It appears that this protuberance is the so-called parenthesis-like structure, which is a typical feature of the cyst when stained by GMS. It was speculated that this thickened zone of the cell wall was related to excystation of the spore, because a foramen-like structure was found in some sections [51]. Consistently, single pores in the cyst wall of *Pneumocystis* empty cysts were shown, suggesting a mode of excystation for the spores [50]. However, as far as we know, no spore leaving the cyst through these pores has as yet been observed.

Each spore has a nucleus with a double membrane. The thickness of their cell wall is close to the thickness of the cell wall of trophic forms. The outer membrane is easily visible in this stage [40,67], which presents a dense cytoplasm with a circular mitochondrion, few lamellae of well-developed rough endoplasmic reticulum, and numerous ribosomes. Spores may present three morphological patterns: a spherical form; a banana-shaped electron-dense form; and an irregular, varying form filling up the cyst [51,67]. The significance of these variations is not understood. Mature cysts containing banana-shaped electron-dense spores were detected in the bronchial lumen by using TEM, suggesting they could gain the external environment in air and be transmitted to other hosts by the airborne route [10,49]. PcP may be experimentally transmitted to deeply immunodepressed hosts (SCID mice, nude rats, corticosteroid-treated rats) by inoculating them by

the nasal or tracheal route with viable parasites (see Ref. 26 for review). Inoculum can be either freshly extracted parasites from infected rodents or parasites developing in *in vitro* cultures [32,93]. But as inoculum is usually a mixture of vegetative, sporocytic, and cystic forms, we do not know which *Pneumocystis* stage is responsible for the infection. Likewise, in natural conditions, the infective stage of *Pneumocystis* remains totally unknown, though *Pneumocystis* DNA has been detected in air [94,95] and in water [96].

VII. Relationships of *Pneumocystis* Organisms to Alveolar Epithelial Cells

Pneumocystis organisms are able to proliferate inside the pulmonary alveolus, where they attach specifically to type I alveolar epithelial cells (Fig. 8). Adherence of *P. carinii* to type I pneumocytes is the first step in the initiation of infection by the parasite. Ultrastructural studies have revealed a close *in vitro* attachment of trophic forms (Fig. 7) and, to a less important extent, of sporocytic and even cystic forms to monolayer cultured fibroblastic or epithelial cells [93,97–99]. These observations suggest that *Pneumocystis*–host cell interactions are required for parasite proliferation.

In parasitized lungs of humans, rabbits, mice, or rats as well as in explant cultures of parasitized rabbit lung [98–100], *Pneumocystis* attachment was observed only on type I epithelial alveolar cells. Small trophic forms still without filopodia were already able to attach to the host cell plasma membrane by a regular, smooth adherence surface (Fig. 6). Larger, presumptive vegetative forms developed more intricate contact, but without fusion of cell membranes (Fig. 8). The attachment surface showed marked infolding. Numerous clear membrane-bound vesicles, which apparently released their content into the parasite–host cell area (Fig. 8), were observed in the adjacent cytoplasm of the host cell. Sometimes narrow, flexuous trophic-form filopodia developed in the lung's intercellular spaces penetrating more and more deeply and adhering to epithelial alveolar cells [98]. Numerous vesicles in the cytoplasmic area next to the attachment zone were also observed in these cells. An outstanding observation was the development of short, intracellular trophic-form filopodia with somewhat bulging apical tips that anchor the parasite to the host cell's cytoplasm without disrupting the plasmalemma [98].

As in other pathogenic fungi where fungal cell wall receptors interact with host proteins, such as fibronectin, laminin or fibrinogen, cell surface molecules of *Pneumocystis* organisms could play a similar role in parasite binding to the host cell. For instance, the *Pneumocystis* attachment to lung epithelial cells is mediated by fibronectin-binding integrins [91,99,101], and specific fibronectin-binding receptors were characterized in the *Pneumocystis* MSG [91]. Consistently, parasite attachment rates seemed to be higher when host cells produce an

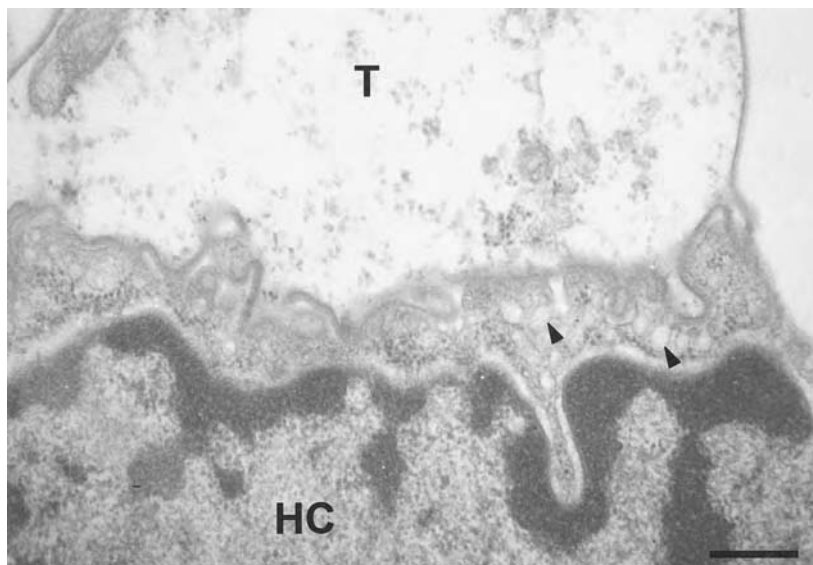


Figure 8 Trophic form (T) of rabbit-derived *Pneumocystis* in vivo, attached to an alveolar cell of type I (HC) with numerous clear membrane-bound vesicles (arrowheads) in the adjacent cytoplasm. Bar = 0.5 μ m. (Adapted from Ref. 98.)

extracellular matrix richer in fibronectin [32,99,102]. Vitronectin, mannose, and laminin are also involved in *Pneumocystis* attachment to epithelial cell lines [103–105]. Also, macrophage mannose receptor [106], fibronectin [107], and probably the complement receptor of the macrophage are involved in the adherence of *P. carinii* to this cell. However, *Pneumocystis* binding to the macrophage does not result in either activation of this cell or parasite phagocytosis. The internalization and degradation of the parasite are enhanced by opsonisation with specific antibodies and complement [108].

Attachment or other interactions between *Pneumocystis* organisms and alveolar epithelial cells are surely involved in PcP pathogenesis. The binding of *P. carinii* organisms may exert direct growth impairment on the epithelial cell [109]. However, recent in vitro experiments indicate that *P. carinii* attachment does not alter metabolic, structural, or barrier functions of alveolar epithelial cells [110]. These results were consistent with our own in vivo TEM observations, revealing that *Pneumocystis* attachment does not seem to alter type I alveolar epithelial cells. On the contrary, *Pneumocystis* attachment induces an apparent activation of their plasmalemmal vesicular system [98,111], which could facilitate metabolic exchanges with parasites (Fig. 8).

In fact, inflammatory cells or their mediators, such as tumor necrosis factor alpha (TNF- α), are required to induce the epithelial injury associated with PcP. It begins with an increase of the alveolar-capillary membrane permeability [112] that may explain, at least in part, the interstitial edema. The injury to the alveolar epithelium leads ultimately to denudation of the basement membrane. Then, a host reparative response takes place by hypertrophy and hyperplasia of type II epithelial alveolar cells [112,113]. These processes lead to the filling of alveolar spaces with masses of parasites, alveolar macrophages, desquamated epithelial alveolar cells, and eventually polymorphonuclear leukocytes. Thus, these mixed cells, at varying degrees of damage, remain clearly identifiable at the ultrastructural level, appear at the light microscopy level as the pneumocystosis typical foamy eosinophilic material.

VIII. Interactions of *Pneumocystis* Cells with Pulmonary Surfactant

Since *Pneumocystis* organisms dwell on the surface of the alveolar epithelium, which is lined by the aqueous subphase of surfactant, interaction of parasite cells with surfactant components are surely relevant to the parasite's cell biology. Several studies have demonstrated surfactant abnormalities associated with PcP in AIDS patients. Amounts of phospholipids are decreased and the lipid composition is altered [114–117]. A reduction in diacylglycerophospholipid [115], a decrease in phosphatidylcholine, and an increase in lysophosphatidylcholine have been reported [114,116]. Furthermore, Escamilla et al. [114] have shown in AIDS patients that changes in surfactant occurred early, with very low levels of *Pneumocystis* organisms and before the pneumonia set in. High levels of SP-A, which were correlated with the number of *Pneumocystis* organisms, were detected in patients with severe PcP [118]. Limper et al. [119] have reported similar changes for SP-D. Interestingly, it was recently reported that SP-A plays a role in host defense against *Pneumocystis*, perhaps by functioning as an opsonin [120]. Opposite changes were found recently in hydrophobic SP-B and SP-C [121,122]. Studies performed by using corticosteroid-untreated PcP models, such as rabbits or SCID mice [121,123], have clearly shown that surfactant changes were linked to *Pneumocystis* growth. As in humans, these changes occurred early, *before* a noticeable proliferation of the parasite [123]. Indeed, *Pneumocystis* organisms may alter the amount and type of surfactant by inhibiting the synthesis and the secretion of phosphatidylcholine from rat alveolar type II cells [124]. Likewise, the parasite is apparently able to enhance the expression of SP-A and SP-D mRNAs, two SPs that can bind *Pneumocystis* [89,90] through MSG, and enhance *Pneumocystis* attachment to alveolar macrophages [119,125]. Thus, contrary to recently reported observations in reconstituted SCID mice [126], many data show that changes in *Pneumocystis*-induced surfactant result, at least partly, from an

early, direct alteration of surfactant metabolism by the parasite. On the whole, *Pneumocystis*-induced changes in surfactant could favor the progression of the *Pneumocystis* infection. In *Pneumocystis*-infected hosts a clear improvement of pulmonary function was consistently obtained by surfactant replacement [127–130]. Moreover, exogenous therapeutic surfactant preparations did inhibit the parasite's growth in vitro in short-term cocultures [123], suggesting that abnormal surfactant changes associated with PcP could be critical factors for the proliferation of *Pneumocystis*.

IX. Morphological and Biological Differences Among *Pneumocystis* Species

The cluster of marked host species–related genomic, karyotypic, and antigenic differences among *Pneumocystis* populations [4,131,132] probably expresses the adaptation of specific parasite lines to different mammals. This process surely involved speciation, and the status of *Pneumocystis* natural populations therefore conforms to the biological definition of species [8,18,49,132,133]. Consistently, a close host species–specificity was shown in *Pneumocystis* isolates from several host mammals (see Ref. 134 for review) including humans [16]. Beside selective infectivity, *Pneumocystis* species show morphological and biological differences at the cellular level. They are briefly reviewed in this section.

Morphological divergence among *Pneumocystis* organisms from different mammals was revealed relatively recently. Indeed, whereas *Pneumocystis* species showed little difference at the light microscopy level, a clear host species–related divergence was found at the ultrastructural level [9,11]. Most objective divergence involves filopodia (Fig. 9). These typical structures of *Pneumocystis* presumptive vegetative forms are markedly more numerous, thin, and tree-like in *Pneumocystis* organisms from mouse than in those from rabbit, human, or macaque [7,9–11]. Filopodia of rat-derived organisms were also found to be smaller than those from rabbit-derived *Pneumocystis* [11]. Furthermore, the density and diameter of membrane-limited electron-dense cytoplasm granules were found to be respectively higher and larger in mouse than in rabbit-derived *Pneumocystis* organisms. Additionally, mouse-derived parasites showed globular, electron-dense, bulbous dilatations at the tips of filopodia. These swellings were not found in parasites from other mammal host species [11].

Differences in in vitro behavior were reported among *Pneumocystis* isolates from rat and mouse [99]. Thus, rat-derived *Pneumocystis* organisms seemed to have a higher capacity for attaching in vitro to target cells than mouse-derived parasites. Furthermore, the in vitro attachment of rat *Pneumocystis* seemed more sensitive to pentamidine or cytochalasin-B than attachment of mouse-derived organisms [99]. But it is perhaps the growth rate of *Pneumocystis* organisms that best showed the importance of the biological divergence among *Pneumocystis* organisms from different mammals. Actually, the doubling time of *Pneumocystis*

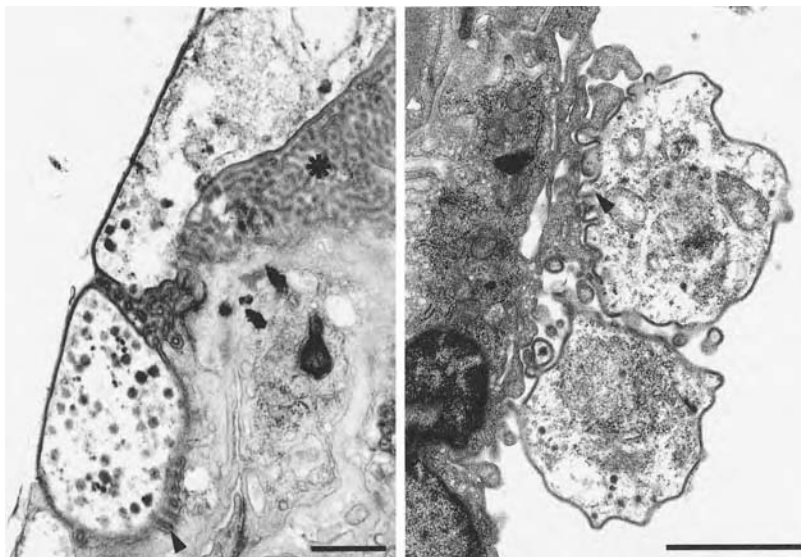


Figure 9 Ultrastructural differences between mouse- and rabbit- derived *Pneumocystis* organisms. On the left, there are mouse-derived, attached trophic forms that display numerous thin filopodia (arrowhead) that appears tree-like in cross section (asterisk). On the right, there are rabbit-derived trophic forms with typical thick filopodia (arrowhead) attached to the lung epithelium. Bar = 1 μ m.

organisms developing in the host lung was highly variable in terms of the host species: it was 1.7 days for rabbit-derived *Pneumocystis*, 4.5 days for rat-derived *Pneumocystis*, and 10.5 days for parasites growing in the SCID mouse lung [57]. On the whole, distinct host species–linked ultrastructural or biological differences among *Pneumocystis* organisms from different mammals strengthen the view of multiple species existing within the *Pneumocystis* genus [18].

X. Conclusions

Up to 1989–1990, neither a Golgi complex nor a lysosomal system was known in any stage of *Pneumocystis* organisms and only fragmentary ideas of the other parasitic cellular structures were available. Improvements in methods for obtaining well-preserved *Pneumocystis* samples for ultrastructural study—associated with the application of cytochemistry, immunolocalization, serial sectioning, and computer-aided 3D reconstruction methods—have revealed the true nature of the *Pneumocystis* cell.

Thus, over the last 12 years, it has been shown that *Pneumocystis* organisms have a highly complex endomembranous system, which includes RER and SER,

Golgi vesicles, lysosomes, and complex endoplasmic saccular structures (ES₁ and ES₂). Endomembranous structures and the Golgi complex, which are not highly developed in trophic forms, develop to considerable complexity in the intermediate sporocyte, suggesting that they are involved in the synthesis of sporocytic cell wall compounds.

An adequate knowledge of the ultrastructural morphology of *Pneumocystis* organisms is obviously essential to fully understand their basic biology, the kind of relationships they can establish with host alveolar cells, and therefore PcP pathophysiology. Furthermore, the study of the fine morphology of *Pneumocystis* organisms should provide valuable phenotypic support to the description of *Pneumocystis* species, though the recognition of species will rely predominantly on sequence divergence and host specificity [18,125].

Pneumocystis molecular taxonomy, in revealing a new heterogeneous group of eukaryotic parasites widely spread in ecosystems, has opened up a virgin field for research in basic biology. In this perspective, it is clear that research on the cell biology of these new organisms is only just beginning.

List of Abbreviations

ES₁, ES₂: endoplasmic saccules of type 1 or 2, respectively
 GMS: Gomori-Grocott's methenamine silver nitrate
 HES: hematoxylin-eosin-saffron
 IMP: intramembranous proteins
 KARD: karyotype and restriction display
 Msg: major surface glycoprotein
 MSG: family of Msg molecules
 PAS: periodic acid-Schiff
 PcP: *Pneumocystis* pneumonia
 Prt-1: subtilisin-like serine protease 1
 PRT: family of Prt molecules
 RER: rough endoplasmic reticulum
 SEM: scanning electron microscopy
 SER: smooth endoplasmic reticulum
 SP A-D: surfactant proteins A to D.
 TBO: toluidine blue O (TBO), or orthotoluidin blue
 TEM: transmission electron microscopy
 TPPase: thiamine pyrophosphatase
 ZIO: zinc iodide-osmium tetroxide

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4

Surface Antigens

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I. Introduction

Studies on surface antigens constitute a major part of the biochemical research performed on *Pneumocystis*. Surface antigens have garnered extensive attention for two reasons. First, they are of fundamental interest because they reside at the interface between host and pathogen and so would seem to be crucial for both the pathogen and the host response to its presence. Second, they are accessible because they are highly abundant proteins that are concentrated on the cell wall/surface, a part of the cell that is relatively easy to isolate.

While it has been possible to obtain surface antigens prepared directly from *Pneumocystis* organisms, this approach has been nevertheless hampered by the limited quantity and quality of the organisms that can be obtained from infected lungs, which is the only environment known to support luxuriant growth of this fastidious fungus. A robust culture system has yet to be formulated. Fortunately, just as biochemical approaches were reaching their limit, a gene encoding a surface antigen protein was isolated. Cloning this gene allowed the first major surface antigen of *Pneumocystis carinii* to be defined as a protein sequence. An unexpected dividend of acquiring this gene was the subsequent discovery that it is a member of a family of genes that appears to be devoted to generating surface

antigen variation similar to that seen previously in protozoan parasites and bacterial pathogens. *Pneumocystis* was the first fungus discovered to possess such a gene family. The surface antigen encoded by this family of genes has been given a variety of names, but is most often called either major surface glycoprotein (MSG), or glycoprotein A (gpA). For the sake of brevity, it is called MSG herein.

MSG is the best-studied *Pneumocystis* antigen, and the structure and function of the MSG gene family have been extensively investigated in one species of *Pneumocystis*, *P. carinii*, which is found in rats. Consequently, most of this chapter is devoted to MSG and *P. carinii*. Also reviewed are data pertaining to MSG from other *Pneumocystis* species and to other surface proteins that have been discovered either because they are known to be antigenic during infection or via studies stemming from isolation of a gene. Carbohydrate antigens are discussed, but only in the context of modifications to MSG and other surface proteins. The literature pertaining to the binding properties of *Pneumocystis* organisms is too extensive to be covered here except when it pertains to specific proteins. Extensive review of the effect of the microbes on the host, while no doubt mediated by binding interactions, is also beyond the scope of this review.

II. Identification of Surface Antigens

A. Overview

Molecules that are attached on the outside of *Pneumocystis* organisms and recognized by the host immune system were first identified by chemical modification studies, which allowed surface proteins to be defined as bands on denaturing polyacrylamide gels. The nature of material in the bands was characterized by using proteases, deglycosylases, and lectins. In later work, antibodies were used in conjunction with physical analysis and microscopy to characterize surface proteins. The predominant surface proteins are large, abundant glycoproteins that tend to be recognized by antibodies present in infected hosts. Ultimately, genes encoding surface antigens were cloned by using antibodies to screen expression libraries. Analysis of cloned genes defined surface antigens as protein sequences and led to the discovery of additional protein surface antigens.

B. Surface Labeling

When biotin-conjugated lectins were used to detect glycoproteins in electrophoretically separated proteins of *P. carinii*, multiple entities exhibited intense reactivity with both concanavalin A and wheat-germ agglutinin (1). These lectins also stained alcohol-fixed *P. carinii*. Electron micrographs showed that concanavalin A bound to the outer surface of *P. carinii* organisms and to tubular extensions emanating from the exterior surface.

Further surface labeling experiments served to better define the proteins on the exterior of *P. carinii* organisms. *P. carinii* were exposed to either radioactive iodine (2) or biotinylation (3). Both techniques identified molecules that migrated in denaturing polyacrylamide gels at apparent molecular weights ranging between 115 and 120 kDa. These molecules were destroyed by protease treatment and migrated faster after treatment with deglycosylating agents, indicating that the major surface antigen comprises one or more glycoproteins (3). Surface labeling with lectins indicated that the carbohydrates on the surface contain mannose, N-acetylglucosamine, and N-acetylgalactosamine (4).

C. Studies with High-Titer Antibodies

Studies with antibodies have identified proteins with properties resembling those of protein detected by surface labeling (5–16). High-titer antibodies against surface antigens have been raised by standard methods. In some studies, semipurified material was prepared either by extracting cell walls or by affinity chromatography using lectins to enrich for glycoproteins. The material obtained was used to elicit antibodies in rabbits or mice. In other cases, whole *Pneumocystis* organisms were used to elicit a panel of mouse monoclonal antibodies, which was screened to identify antibodies that bind to *Pneumocystis* surface antigens.

To determine the nature of antigens detected by high-titer antibodies, the antibodies have been used in conjunction with treatment with degradative enzymes and chemical modifiers (6). In these studies, susceptibility to epitope degradation was monitored via immunoblotting and immunofluorescence. A 116-kDa band of *P. carinii* was found to be susceptible to proteolytic (e.g., trypsin) and glycolytic (e.g., Zymolyase) treatments but not to a variety of other procedures (e.g., lipase). This moiety reacted strongly with concanavalin A and wheat germ agglutinin, indicating the presence of mannosyl or glucosyl and N-acetylglucosamine residues. Immunofluorescent staining and surface labeling suggested that the 116-kDa antigen was located on the *P. carinii* cell wall. A 116-kDa band of human *Pneumocystis* behaved in similar fashion. Two other strongly immunoreactive bands of *P. carinii*, 45 to 50 kDa, were more resistant to proteolytic enzymes and exhibited poor binding to lectins and low reactivity by surface-labeling procedures. The human *Pneumocystis* 40-kDa band exhibited properties similar to those of the two smaller *P. carinii* antigens. Tanabe et al. also analyzed the major surface proteins of *P. carinii* by immunochemical and biochemical methods. In this study, a large portion of the total proteins migrated at approximately 115 kDa (8). Purified 115-kDa protein (p115) exhibited six isoelectric variants when analyzed by two-dimensional gel electrophoresis. A monoclonal antibody recognized all six variants and reacted with epitopes that were located in the cell wall, indicating that p115 was an immunoreactive surface component. Deglycosylated p115 migrated primarily as a single, more acidic

protein in two-dimensional gels, suggesting that the isoelectric variants were due primarily to differences in glycosylation.

D. Studies with Antibodies from Infected Hosts

Protein antigens of *Pneumocystis* have been identified by immunoblot analysis using antibodies from infected animals and humans. In one study, more than 400 human sera were characterized (17). Greater than 85% of sera specimens from individuals at least 2.5 years of age contained antibodies to a 40-kDa band. Bands of 66, 92, and 116 kDa were also frequently detected. Another group found that the majority of patient sera tested reacted strongly with moieties that migrated either between 110 and 116 kDa or between 55 and 60 kDa. Antigens with apparent masses of approximately 170, 125, and 30 to 32 kDa were also detected with some but not all sera (18). Another laboratory found that sera from rats tended to detect *P. carinii* antigens with apparent molecular masses of 45 and 110 kDa, as well as a mass of material migrating between 49 and 64 kDa. Sera from humans detected bands of 22 and 24 kDa, and between 35 and 45 kDa (19). A fourth group reported that the majority of human sera recognized a 115-kDa antigen (8).

A study of primary *Pneumocystis* infection in rabbits by immunoblotting analysis showed that serum IgG recognized a band of 50 kDa, while IgM recognized a band of 45 kDa (20). Studies in mice examined *Pneumocystis* antigens recognized by antibody-secreting B cells cloned from tracheobronchial lymph nodes isolated immediately following recovery from pneumonia (21). Nearly all of the cloned cells produced antibodies that reacted with an antigen expressed on the surface of mouse *Pneumocystis*. When one of these antibodies was used to probe an immunoblot, a broad range of antigenic material was observed, with the most intense reactivity in the 50- to 65-kDa region of the blot.

There are two principal morphological forms of *Pneumocystis*, which occur together in most infections (22,23). One form, called the trophic form, resembles a yeast cell except that it has a flexible and fragile cell wall. The other form, called the cyst, resembles a fungal spore sac. It has a rigid, thick cell wall, which encloses up to eight presumptive spores. Not much is known about these forms at the molecular level, but a study using immunoblotting with hyperimmune rat sera reported differences between the two forms of *P. carinii* (24).

E. Immunoreactive Proteins of *Pneumocystis* from Different Host Species Tend to Differ

The studies reviewed above commonly reported size differences in the immunoreactive proteins of *Pneumocystis* from different host species. In addition, monoclonal antibodies raised against *P. carinii* tend not to recognize proteins from other *Pneumocystis* species (5,6,9,11,12,14,25) The reason for these differences

was difficult to discern at first because all studies relied on organisms obtained from infected lungs, leaving open the possibility that host-specific factors may have caused the differences in *Pneumocystis* proteins. Such factors may be involved, but they need not be, because it is now known that each host species carries a different species *Pneumocystis* (26). It should be noted that more than one species of *Pneumocystis* can occur in a given host, as is the case for *P. carinii* and *P. wakefieldiae*, which are both found in rats. *P. carinii* and *P. wakefieldiae* exhibit distinct immunoblot reactivity patterns (27,28).

F. Summary of Findings from Direct Analysis of Surface Antigens

The most abundant antigenic protein migrates as though it were between 115 and 120 kDa in mass, although some studies on human *Pneumocystis* found the major antigen to be as small as 95 kDa or as large as 140 kDa. While not as abundant, proteins that migrate as though they are between 40 and 55 kDa in mass are generally found to be very strongly immunoreactive. A number of other bands have been implicated as surface antigens. These include bands with apparent molecular masses of 170, 125, 110, 100, 92, 66, 64, 60, 35, 32, 30, 24, and 22 kDa. This list of antigens might be longer than it should be. Differences between different species of *Pneumocystis* combined with laboratory-to-laboratory differences would tend to inflate the number of antigenic species observed.

III. Genes Encoding Surface Antigens

Studies on surface antigens moved to a molecular level with the cloning of genes that encode these proteins. Such genes have been obtained via two routes: (1) expression cloning, where an antibody was used to screen a panel of bacteria designed to express *Pneumocystis* epitopes, and (2) serendipitous discovery, where genes discovered by other means were later found to encode antigenic proteins. So far, four types of antigen-encoding genes have been described.

A. p55

Pneumocystis carinii contains a major group of antigens that migrates as a broad band of 45 to 55 kDa on denaturing acrylamide gels. This antigen is recognized by serum antibodies from most if not all infected individuals. A cDNA encoding p55 was obtained by screening a cDNA library designed to express *Pneumocystis* epitopes in bacteria. The predicted protein encoded by this cDNA protein is not related to a protein of known function. P55 contains 414 amino acids and a distinctive domain composed of 10 copies of a seven-amino-acid sequence motif rich in glutamic acid residues (29,30). The protein made from the cloned cDNA was recognized by serum antibodies from rats and humans with *Pneumocystis*

pneumonia. The p55 protein was shown to be on the surface, but masked by components of the cell wall that can be removed by beta-1,3 glucanase (31). Antibodies reactive with the *P. carinii* peptide also recognized a protein from human *Pneumocystis* that migrated between 35 to 45 kDa (29).

B. MSG

Expression cloning was also successful in isolating major surface glycoprotein (MSG) genes. Studies on surface antigens had shown that the major *P. carinii* surface antigen was an abundant glycoprotein that migrated as though it were between 115 and 120 kDa in mass (1–3,6–8,11,13,15,27,28,32–36). Using antibodies that recognized this material, several cDNAs were isolated from libraries designed to express *Pneumocystis* epitopes in bacteria. The first such cDNA of interest was obtained from ferret-derived *Pneumocystis* (37). Experiments on *P. carinii* identified several cDNAs that encoded related proteins (38,39). The relationship between MSG and the proteins encoded by the cDNAs was confirmed for *P. carinii* by showing that peptide sequences from MSG matched those encoded by one or more of the cDNAs (38).

Analysis of cDNAs showed that a *P. carinii* population present in a single rat lung can express mRNAs encoding many different isoforms of MSG (40). These cDNAs were found to originate from different MSG genes. The MSG gene family has been extensively studied and appears to be dedicated to generating surface variation. This subject is reviewed in detail below.

Expression cloning also showed that monoclonal antibodies that recognize the MSG band on an immunoblot bind to epitopes encoded by MSG genes (41). This work established the molecular identity of the material previously detected by the surface labeling studies described above. In a few cases, a monoclonal antibody has been observed to react with a protein not related to MSG (42). Such phenomena are not unexpected and underscore the importance of obtaining a thorough profile of monoclonal antibody targets.

C. PRT1

A third group of surface antigens was discovered in the course of sequencing MSG genes and flanking DNA. In *P. carinii*, the PRT1 (also known as KEX) multigene family encodes isoforms of a subtilisin-like serine protease (43–46). The discovery of this gene family was a surprise because other fungi have only a few genes encoding this type of protease, known as kexins, which function primarily in the processing of preproteins as they make their way to the cell surface (47). The proteins encoded by the different *P. carinii* Prt1 genes share some motifs, such as those that constitute the active site, but differ elsewhere. This variation suggests that PRT1 proteases may appear on the surface of

P. carinii. In addition, copies of the Prt1 gene family are closely linked to genes encoding different isoforms of the *P. carinii* major surface glycoprotein.

Immunohistochemical, immunofluorescent, and immunoelectron microscopy studies have localized epitopes of this protease to the organism surface (44,45). One antiserum raised against pure PRT1 recognized a *P. carinii* protein band migrating between 42 and 52-kDa (44). Hence, this protease may be a component of the 40 to 55 kDa antigenic material detected previously via studies with serum antibodies present in infected rats (see above). A gene encoding a related protease has been described in mouse *Pneumocystis*, but in this species the gene appeared to be present in only one copy (48). More information about the PRT1 gene family is available and is discussed below.

D. MSR

A second example of a probable surface antigen that was found indirectly is the group of proteins called MSR, which are related to the major surface glycoproteins (49). MSR genes have been called by other names, including “type II MSG” (50) and “variant MSG” (51). However, if MSG genes are defined by their association with the upstream conserved sequence (UCS), which seems appropriate, because MSG open reading frames (ORFs) are not expressed unless they attach to the UCS (see below), then MSR genes should not be called MSG genes because they do not attach to the UCS locus. The number of MSR genes in the genome is not known, but they appear to be located on at least 13 chromosomes (45). Like PRT1 genes, MSR genes are located next to MSG genes, but MSR genes contain an intron, while MSG genes do not (49–51).

MSR proteins have been characterized to a modest extent. Antibody raised against MSR epitopes detected products in molecular masses ranging from 90 to 115 kDa (50,51). Approximately 10% of the apparent mass of MSR appears to be due to N-linked glycosylation (51).

E. PRT1, MSR, and MSG Genes Are Organized as Clusters at the Ends of Chromosomes

Clustering of MSG genes was recognized soon after the discovery of the gene family, because previous work had already identified and characterized repeats that turned out to be MSG gene clusters. DNA hybridization experiments showed that the cDNAs isolated using the anti-MSG antibody are related to the 3–1 family of repeated DNA sequences, which had previously been shown to have approximately 100 members occurring in clusters distributed throughout the genome (52). Sequence analysis of a 3–1 family clone revealed the presence of two open reading frames (ORFs) encoding two related 120-kDa peptides (53). Neither of the two ORFs in this clone contained introns. Lack of introns was later found to be a feature of all ORFs encoding MSGs. By contrast, other *Pneu-*

mocystis genes generally contain multiple introns. The absence of introns in MSG genes may reflect the process that generated the family. One possibility is that the progenitor MSG gene had introns, but that the genome gained many additional copies of MSG genes via reverse transcription of mRNAs, followed by integration of the resulting cDNAs. Another mechanism that may have helped increase the number of MSG genes is unequal reciprocal recombination reactions between two MSG genes. Indeed, unequal reciprocal recombination is probably still happening, because MSG ORFs are oriented in head-to-tail fashion, spaced only about 500 bp apart (53). MSG genes are linked to telomeres, which would help facilitate unequal reciprocal recombination reactions (54). Such recombination reactions happen most often when the participants are very nearly identical in sequence. Hence, recombination can be inhibited by introns, which tend to diverge more than protein-encoding regions of genes.

The first indication of clustering of MSG, MSR, and PRT1 genes came from analysis of a 11,370-bp chromosomal DNA segment (43). This DNA segment contained three ORFs, which were named MSG99, MSG100, and ORF-3. MSG99 and MSG 100 were clearly related genes. Proteins encoded by MSG99 and MSG100 shared 39% protein sequence identity, but the virtual MSG99 peptide was substantially shorter due to the absence of a contiguous segment comprised of approximately 333 amino acids. MSG99 turned out to be an MSR gene. ORF-3 encoded a putative 47,820-Da protein that had little sequence similarity to MSG molecules. ORF-3 turned out to be a PRT1 gene. Recently, several more MSG-MSR-PRT1 gene clusters have been sequenced (see Fig. 1). Linkage of PRT1, MSR, and MSG genes in the same orientation suggests that they may have evolved from a common ancestral gene via gene amplification caused by unequal reciprocal crossing over. Tandem repeats also suggest the possibility of coordinated transcription. Whether PRT1 and MSR genes are regulated or not has not been determined, but it is clear that more than one can be expressed in a single population of *P. carinii* (44–46,49–51,55).

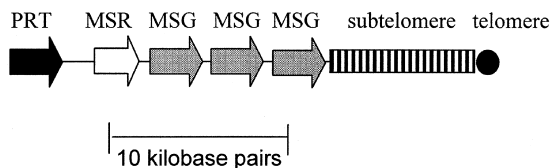


Figure 1 Map of a cluster of telomeric surface antigen genes. This cluster is from one end of the smallest chromosome in the genome. Each end of each chromosome appears to have a cluster of surface antigen genes. Different clusters can have different structures, but in all known cases all of the genes are oriented towards the subtelomere, as shown. Most clusters have at least one copy of the three-gene repeat PRT-MSR-MSG.

IV. Structure and Function of the MSG Gene Family of *Pneumocystis carinii*

It should be noted at the outset that the collection of related DNA segments comprised by the “MSG gene family” are probably not genes per se because they are not transcribed in situ. All evidence to date indicates that to be transcribed, an MSG ORF must first become linked to a unique site in the genome, the expression site (see below). According to the expression site model, MSG ORFs are not genes in the strict sense but rather constitute a reservoir of DNA sequences, any and all of which may be used to form genes. Nevertheless, it seems justified to refer to MSG ORFs as genes because, as far as we know, all of the MSG ORFs in the genome have the ability to associate with the expression site and to be expressed. At any rate, most of the literature on the subject refers to MSG ORFs as MSG genes, and this convention is followed herein.

A. Organization and Number of MSG Genes

MSG genes appear to be on all chromosomes, because all of the chromosome-size DNA bands resolved by pulsed-field gel electrophoresis (PFGE) hybridize to DNA probes made from either a single MSG gene or cDNA (38,39,53,56,57). The exact number of *P. carinii* chromosomes is not known, but PFGE usually resolves at least 15 bands. There may be as many as 19 chromosomes, however, because three bands stain more intensely with DNA stains than others do, suggesting that these bands contain two chromosomes each (58–61).

As far as is known, all members of the MSG gene family reside at the ends of chromosomes. The initial evidence for telomere linkage came from a cloned DNA segment that began with the 3' end of an MSG gene and ended with a sequence known to be at subtelomeric regions of the *P. carinii* genome (54). Confirmation that telomere linkage is a general feature of the gene family was obtained by demonstrating that MSG genes were highly sensitive to degradation when intact *P. carinii* chromosomes were digested with an exonuclease (62). More recently, several full arrays of MSG genes have been cloned as cosmids and completely sequenced. Each of the three sequenced arrays begins in a unique sequence that resides at the end of a chromosome, and each ends in either a telomere or a subtelomere. Figure 1 shows an example of one such gene cluster.

The number of MSG genes in the *P. carinii* genome is not clear. Data from chromosome ends suggests that each end carries either 2 or 3 MSG genes. If there were the same number of MSG genes at the ends of 19 chromosomes, the number of genes per genome would be between 76 and 118. These numbers agree well with an early estimate, obtained by DNA hybridization, of 100 copies per genome (53). However, this was probably an overestimate, because MSR genes would have been detected along with MSG genes. Unpublished sequencing data

indicate that the MSG gene family contains at least 80 members, but this is a minimum size for the family, because new genes are still being found.

Of the 80 or more MSG genes that appear to be carried in the *P. carinii* genome, only one appears to be expressed in a given *P. carinii* organism at a given time. This gene resides at a unique telomeric locus called the UCS, so named because it contains the upstream conserved sequence found at the 5' end of all messenger RNA molecules containing MSG ORFs. The evidence supporting the expression site model is discussed below.

3. Regulation of MSG Gene Expression

Discovery of the UCS

Expression cloning yielded *P. carinii* cDNAs encoding several different MSGs (38). Additional studies established that multiple MSG ORFs can be represented by messenger RNA molecules in a population of *P. carinii* obtained from a single rat (40,56). Two alternative explanations for these data presented themselves. One possibility was that more than one MSG was expressed in a given *P. carinii* organism at a given time. Alternatively, expression of multiple MSG genes within a population of *P. carinii* could have been caused by different genes being expressed in different organisms in the population.

At this stage of the investigation, it was unclear whether it would be possible to distinguish between the two alternative explanations of the cDNA data, considering the lack of a system for culturing clonal populations of *P. carinii*. Fortunately, basic studies on the structure of messenger RNAs encoding MSGs revealed a feature that allowed progress in the absence of a robust culture system. The first 429 bases were discovered to be the same in 21 of 21 cDNAs, even though these 21 encoded different isoforms of MSG (56). The presence of this 429-bp sequence on MSG mRNAs was confirmed by another group using a similar approach (63).

The first 405 bp of the 429-bp sequence at the beginning of MSG mRNAs was named the type I UCS (56). Only the last 24 bp of these 429 bp was present in known MSG genes. These 24 bp became known as the CRJE and encode the peptide sequence MARPVKRQ, which may be the site of protease cleavage suspected to remove the amino terminus of the protein (see below). A few cDNAs had a different sequence at their 5' ends. This sequence was named the type II UCS (56). Subsequent data showed that only the type I UCS is associated with MSG genes, and that the type II UCS is part of what we now call MSR genes (see below). Therefore, herein, "UCS" refers to what was originally called the type I UCS.

The UCS was mapped in the genome by hybridizing a copy of it to chromosomes separated by PFGE. Only one of the 15 chromosomes resolved by PFGE hybridized (56,63). The localization of the UCS to a single PFGE band suggested

that it might be unique in the genome. Results of quantitative hybridization experiments supported this hypothesis (62).

Different MSG Genes Can Be Linked to the UCS

To determine the structure of the DNA encoding the UCS, genomic clones were isolated. Several lambda clones carrying the UCS were obtained by screening genomic libraries from *P. carinii* with a UCS DNA probe (56,62–64). All of these clones contained an MSG gene attached directly to the UCS, with a CRJE in between. However, each of the cloned UCS loci contained a different MSG gene attached to the UCS. The UCS-linked MSG gene was followed by other MSG family members, showing that the clustering of MSG genes previously observed also occurs at the UCS locus. It was expected that the UCS would be telomeric because it is linked to MSG genes, which are telomeric. Experiments with exonuclease-digested chromosomes showed this to be the case (62).

In a given *P. carinii* organism, the UCS locus can be linked to only one MSG gene. Yet studies on populations of *P. carinii* showed that numerous UCS-linked MSG sequences were present. These data suggested that different organisms in the population have different MSG genes at the UCS locus. Mapping of restriction enzyme cleavage sites downstream of the UCS produced results consistent with this hypothesis (62). Further analysis of the UCS-MSG junction was performed by amplifying this region of the genome using the polymerase chain reaction (PCR). The junction was found to be heterogeneous in each of 37 *P. carinii* populations analyzed (65). Furthermore, the UCS-MSG junction appeared to be different in each of the 37 *P. carinii* populations. The large variety of MSG genes adjacent to the UCS locus was consistent with the previously observed presence of mRNAs encoding different MSG isoforms, yet each starting with a copy of the UCS.

By contrast with the heterogeneity of the region downstream of the UCS locus, the region upstream of this locus did not show evidence of polymorphism when subjected to restriction enzyme cleavage, which was capable of mapping the cleavage sites up to 10 kilobases (kb) upstream of the UCS (62). Recently, three cosmid clones were isolated that each carry approximately 20 kb upstream of the UCS. Again, this region appeared to be the same in all three clones (Keely and Stringer, unpublished). Further evidence for a single UCS locus came from analysis of *P. carinii* obtained from rats that had been inoculated with a low dose of the microbe. In these studies, which are described in the following paragraph, a single UCS-MSG junction was present. Hence, all of the evidence indicates there is only one UCS locus. Nevertheless, this issue will not be fully resolved until we have characterized both ends of the chromosome carrying the UCS.

Most of the results described above were obtained by analyzing *P. carinii* obtained from rats that develop *Pneumocystis pneumonia* (PcP) spontaneously

upon immunosuppression while kept in a room with animals that already had PcP (the natural transmission system). Therefore the high degree of heterogeneity at the UCS could have been due to rats being infected by multiple organisms that have different MSG genes at the UCS. This hypothesis was tested by experiments in which rats were infected by inoculation with either a large or small number of *P. carinii* from a population in which the UCS locus was very heterogeneous. In rats that received the high dose (10^7 *P. carinii*), the UCS locus had the same complex structure as in the population from which the inocula were drawn. By contrast, in rats that received the low dose (10 *P. carinii*), the UCS locus in the *P. carinii* recovered from the inoculated rats was drastically simplified. In some rats, a single MSG was attached to the UCS in 90% of the organisms in the population (66,67). These data showed that heterogeneity at the UCS locus can be reduced and suggested that the reduction was due to starting the infection with a single organism. Similar results were obtained with rats that were carrying latent *P. carinii*, which were presumably acquired shortly after birth (Keely and Stringer, unpublished). The observation of less heterogeneity at the UCS locus in inoculated and latently infected rats suggests that the high degree of heterogeneity at the UCS in rats infected by being constantly exposed to rats with PcP (natural transmission) was due to infection by multiple organisms that have different MSG genes at the UCS.

The UCS locus is distinctly variable compared to the rest of the *P. carinii* genome. Populations of *P. carinii* have been compared at loci other than the UCS, but sequence differences either have not been observed or have been limited to single nucleotide positions (68).

The UCS Locus Appears to Control Transcription

Three findings suggested that the UCS locus controls transcription of the MSG gene family: (1) the UCS resides at a single locus in the genome; (2) mRNAs encoding different MSGs begin with the UCS; and (3) all but one of the MSG genes in the genome do not begin with a copy of the UCS. The simplest explanation for these findings is that MSG genes must be attached to the UCS to be transcribed and that different MSG genes can occupy the UCS locus in different organisms. Such a system would work to both restrict expression to a single MSG per organism and to allow an organism to switch the MSG it expresses. It is easy to imagine how the switches could take place, because recombination is known to be capable of moving DNA sequences in the genome. Movement into the UCS locus would simultaneously switch on the newly arrived gene and switch off its predecessor (see Fig. 2).

While the expression-site model could explain all three observations listed above, another possible mechanism of MSG mRNA synthesis merited consideration. *Trans* splicing, which occurs in the kinetoplastida, is a process known to

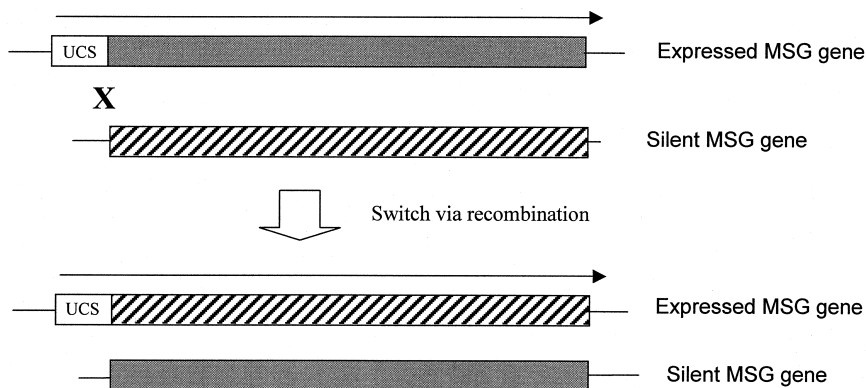


Figure 2 Cis model for regulation of transcription of the MSG gene family. The UCS is the only site in the genome that can transcribe an MSG gene. Therefore, changing the MSG gene at the UCS simultaneously terminates transcription of one gene and activates another.

generate diverse mRNAs that all start with the same 5' end. In *trans* splicing, the sequence that eventually becomes the 5' leader is transcribed from a separate locus and then added to pre-mRNAs by splicing in *trans*—i.e., between two RNA molecules (69). *Trans* splicing would be expected to produce mRNAs for which there is no corresponding UCS-linked MSG gene. Therefore it was possible to test the possible role of *trans* splicing by comparing the structures of the UCS-MSG junctions in mRNAs to those in the genome. Six populations of *P. carinii*, each from a single rat, were studied. All of the populations studied had the same UCS-MSG junctions in mRNAs and the genome (65). These data are not consistent with the *trans* splicing hypothesis.

The UCS Appears to Be Involved in Translation and Transport of MSG

The UCS has a translational start codon near its 5' end (56,63). The start codon is followed by a sequence encoding a putative signal peptide that could serve to direct the MSG into the endoplasmic reticulum (ER), from whence it would presumably be sent to the cell surface. There is evidence that translation starts in the UCS. UCS epitopes are present on a large protein (160,000 Da) containing both UCS and MSG epitopes (70). Hence, the peptide encoded by the UCS appears to be part of an MSG protein precursor. The UCS epitopes were not present on the mature MSG isolated from the cell surface, suggesting that the UCS peptide is removed from the UCS-MSG precursor by a protease. The fate of the UCS after cleavage is not known. If it is removed after the precursor

reaches the surface, which is suggested by the presence of the PRT1 protease on the surface (see below), it may survive as a free unit. There is no evidence either for or against the persistence of free UCS peptides, but the high degree of polymorphism in the genomic copy of the UCS suggests that evolution has selected against uniformity in this peptide, which suggests that it may be a target for the immune system (62).

Although it has not been possible to test the functionality of the UCS peptide in *P. carinii*, this peptide appears to be needed for trafficking MSG into the endoplasmic reticulum (ER) in insect cells (70). This trafficking function was demonstrated by expressing a gene encoding a UCS-MSG protein in insect cells in the presence or absence of tunicamycin, an inhibitor of glycosylation. When made in cells in the absence of tunicamycin, the UCS-MSG protein migrated more slowly than it did when made in cells incubated with tunicamycin, indicating that the nascent polypeptide is glycosylated, which implies that it goes into the ER and Golgi. By contrast, a gene that encoded only an MSG ORF expressed protein that was not glycosylated in insect cells.

These data suggest that the UCS is necessary and sufficient to direct the nascent polypeptide into the compartments known to be traversed by proteins bound for the cell surface. If the behavior of MSG transcripts and precursor peptides is the same in *P. carinii* as it is in insect cells, then MSG genes that are not linked to the UCS would not be expressed as surface proteins, even if such genes were to be transcribed to produce RNAs lacking a 5' UCS. Hence, the UCS appears to exert control over MSG transcription, translation, processing, and transport.

The UCS and MSG portions of the predicted precursor protein are divided by an invariant amino acid string of eight residues (MARPVKRQ) containing a site (KR) that could be cut by a subtilisin-like protease. Molecular genetic analysis has identified a family of genes in *P. carinii* (the PRT1 family) that encodes proteases which could serve this purpose (44–46,55). At least some PRT1 family members appear to be surface proteins, which may be why they vary in sequence (45,46,55).

Expression of a Specific MSG Is Correlated with the Presence of Its Cognate Gene at the UCS Locus

The UCS locus is the only place in the genome known to support transcription, translation, and transport of an MSG. All indications are that there is a single copy of the UCS locus in the genome. Therefore it is very probable that only one MSG isoform is expressed per organism (Table 1). Studies on the surface protein itself have been limited by technical problems, but the available evidence supports this view. Indirect immunolabeling studies have shown that not all organisms within a population could be labeled with an antibody directed against a subset

Table 1 Features of the MSG Gene Family That Suggest Antigenic Variation

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1. The genome contains up to 100 MSG genes, which encode different proteins.
 2. Because the UCS locus is unique, only one MSG gene can be attached to it in a given cell.
 3. Many MSG family members can be linked to the UCS in one organism or another. Therefore MSG genes must be able to move to the UCS and do so at a rate high enough to generate an extraordinary degree of heterogeneity at this site.
 4. MSG genes attached to the UCS are transcribed to produce mRNAs that begin with the UCS and end with an ORF encoding an MSG isoform.
 5. The UCS sequence in the mRNA is translated to produce a putative UCS-MSG precursor protein.
 6. In insect cells, the UCS part of this protein is needed to send it into the ER and Golgi and ultimately to the cell surface.
 7. Populations of organisms in which a high fraction of cells exhibit a specific MSG-associated epitope have a similarly high fraction of cells that have DNA encoding that epitope at the UCS locus.
-

of MSG isoforms (62,71). In addition, the fraction of organisms labeled by such an antibody varied among populations. Additional evidence of limited MSG expression was provided by immunoblotting studies, which showed that some *P. carinii* populations contained a particular MSG epitope in abundance, and other populations did not (28).

Direct evidence that the UCS locus exerts control over the MSG that is on the cell surface is limited but clear-cut. Study of this issue began with identification of a monoclonal antibody, RA-C11, that recognizes a very small subset of the MSG isoforms encoded in the genome (11). First, the C11 epitope and the nucleotide sequence encoding it (C11 epitope encoding sequence, or C11 EES) were determined (42). Then, populations of *P. carinii* were identified that varied over a range of ten fold with respect to the fraction of cells with the C11 EES at the UCS locus. The same populations were analyzed by immunofluorescence to determine the fraction of cells with detectable C11 epitope on their surface. There was a strong correlation between the proportion of C11 reactive organisms and the proportion of organisms with the C11 EES attached to the expression site (42).

It would appear that the MSG protein on the surface can be switched by changing the MSG gene at the expression site. How this switch is accomplished is not known. Studies on the C11 EES suggested that this sequence can be incorporated by a gene conversion into an MSG gene that is at the UCS (Schaffzin and Stringer, unpublished). If such gene conversion events were to occur, the number of different MSG isoforms that could be formed at the UCS locus would be virtually unlimited. Data consistent with this possibility are emerging as the num-

ber of MSG gene sequences increases. More than 75 different sequences have been observed at the UCS locus. Yet so far, only approximately 25 of these sequences have been found at non-UCS sites. These findings may mean that new sequences are being created at the UCS locus. On other organisms, the homologous recombination system can replace segments of genes with DNA from other loci. MSR genes are similar enough to MSG genes to suggest that they may also be able to contribute to MSG diversity by donating DNA into MSG genes via homologous recombination.

On the other hand, changes can occur in the DNA more than 3 kb downstream of the UCS, suggesting that an entire array of genes can move from the end of a donor chromosome to the UCS via a crossover event.(56,62) It is possible, and seems probable, that both gene conversion and crossing over are used to alter the MSG gene at the UCS locus.

C. Insights into MSG Protein Structure Gleaned from MSG Gene Sequences

Analysis of sequences of MSG gene family members predicts certain properties for the members of the MSG protein family. MSG ORFs all encode approximately 1100 amino acids that sum to a molecular mass of approximately 120,000 Da. MSG proteins are rich in cysteine residues located at the same positions in different isoforms (72). The ORFs of all known MSG genes begin with a sequence encoding the amino acids MARPVKRQ. Other amino acid motifs are conserved as well. However, the predicted protein isoforms of MSG vary in sequence by as much as 35%.

Studies on MSG mRNA molecules indicate that multiple MSG genes are often expressed in a population of *P. carinii* (see below), predicting that MSG prepared from *P. carinii* might contain many different proteins. Therefore data obtained via biochemical and immunochemical analysis of MSG prepared from organisms probably should be presumed to pertain to mixtures rather than to a preparation containing a single protein (1–3,6–8,11,13,15,27,28,32–36).

Despite the probable presence of mixtures of different MSG proteins in material isolated from *Pneumocystis* organisms, it has been possible to use biochemical analysis to discern features common to most or all of these proteins. Direct analysis showed that the proteins are glycosylated. Nevertheless, detailed information about MSG protein structure is lacking. Neither end of MSG has been characterized. Attempts to derive peptide sequence from the amino terminus have been unsuccessful, suggesting that this end may be chemically modified. The carboxyl terminus is also uncharacterized. MSG peptides lack a domain that would be expected to traverse a lipid bilayer. The lack of a transmembrane domain suggests that MSG molecules may be attached to the membrane via an anchor that is added posttranslation. Surface proteins in other organisms use a glycosyl-

phosphatidylinositol (GPI) moiety to anchor themselves in the cytoplasmic membrane. GPI has not been demonstrated to be attached to MSG obtained from *P. carinii*, but studies have shown that GPI can be attached to MSG when this protein is expressed in insect or mammalian cells. However, it is possible that MSG is not membrane-anchored but rather attached to the cell wall.

D. MSG Expression and Antigenic Variation

In other microbes that dwell in mammals, gene families serve to generate antigenic variation. By analogy, MSG might be expected to be involved in generating antigenic variation among the individual microbes that constitute a population of *P. carinii* in the lung of a rat (see Table 1).

Antigen variation can be generated by the following model of MSG expression. A constitutively active promoter resides upstream of the UCS locus, and any MSG gene that becomes attached to it is transcribed. Because the UCS is unique, each organism expresses a single MSG isoform by transcribing the MSG gene that is attached to the UCS. The other MSG genes in the genome are not transcribed because they lack promoters. MSG transcripts are translated starting in the UCS, and a UCS-MSG protein is made and sent into the ER, where it is glycosylated and perhaps attached to a membrane-anchor moiety such as GPI. The MSG then emerges on the cell surface. At some point, either prior to being placed on the surface or shortly thereafter, the UCS peptide is removed by a member of the PRT1 family. The MSG isoform that is on the surface can be changed by changing the MSG gene at the UCS. Such a change can take place either by a reciprocal exchange (crossover) between the MSG gene at the UCS and an MSG gene at some other telomeric locus or by copying part of all of a silent MSG gene (or perhaps an MSR gene) into the UCS-linked MSG gene (gene conversion). Switches at the UCS occur spontaneously, thereby continually generating organisms that vary with respect to surface MSG.

The phenotypic impact of a change at the UCS locus would depend on the fate of the old MSG and on the proportion of the surface that is covered by MSG. Once the UCS-linked gene is changed, the transcripts and protein made from the previous UCS resident might remain for a while, but these molecules would decline in abundance as the cells propagate. To illustrate, after five rounds of cell division, only 3% of the mRNA and protein would be from the passé MSG gene. Of course the outmoded MSG mRNAs and proteins could be removed more rapidly through degradative mechanisms. A change at the UCS would have the greatest impact if the MSG were the only protein on the surface. The constitution of the surface is not known, but at least one other surface protein, PRT1, has been identified. The proteins made from the MSR gene family are probably also on the surface. In addition to these major constituents, the organisms would need to have surface proteins that act as transporters. It would be surprising if

there were not other surface proteins as well. The relative amounts of each surface protein are not known precisely, but when surface proteins were labeled and then analyzed, the majority of the labeled protein migrated on a gel at the rate expected for MSG and MSR (2,35).

Preliminary studies have suggested that switches at the UCS occur no more often than 1 in every 100 organisms, but the actual rate of switching is not known and could be much lower (66). However, even if comparatively few organisms were to switch, such as 1 in 10,000, there would be thousands of variants present by the time the population size reached 1 million. If the majority surface antigen were to be recognized by the host immune system, one or more rare variants would then have the opportunity to emerge as the predominant type. This is the pattern seen in several other pathogens, including African trypanosomes (73,74) and bacteria in the genus *Borellia* (75–77).

Microbes such as trypanosomes use antigenic variation to sustain infection in the face of a strong immune response. By contrast, *P. carinii* is unable to cause *Pneumocystis* pneumonia (PcP) in immunocompetent rats, and disease is seen only when the immune system is debilitated. The same is true in humans. Therefore, it seems that MSG-mediated antigenic variation is not able to overcome the immune response, at least not to an extent that would cause PcP.

Perhaps MSG variation is involved in allowing the microbe to colonize its host. *Pneumocystis* organisms are an extremely widespread and enduring associate of mammals (78–85). At least one member of the *Pneumocystis* genus has been found in every mammal that has been examined, including a large number of animals captured in the wild. It is commonly reported that there are thousands of *Pneumocystis* organisms in the lungs of apparently healthy wild animals (83,84,86–88). Laboratory animals are also frequently colonized (78,79).

The frequent-colonization hypothesis fits with the observation that each host species has its own kind of *Pneumocystis*, suggesting that the microbe and host have coevolved and that the microbe depends on its specific host for survival (89). This suggestion is supported by the fastidiousness of these microbes. They do not thrive in culture and seem to be able to propagate continuously only in the lungs of the host in which they are found. It is reasonable to speculate that the MSG system serves to foster survival of *P. carinii* in the lungs of immunocompetent rats, and that this survival is necessary to assure transmission to other rats.

The evidence in favor of the antigenic-variation model is strong, but indirect. There are still many aspects of the system that remain uncharacterized (Table 2). Immunodetection of MSG epitopes on *P. carinii* would appear to have demonstrated nonuniform expression of these epitopes, but such experiments cannot exclude the possibility that the epitope is present but not detected. Similarly, transcription studies would seem to have established that only one MSG gene is expressed at a time. However, uniform transcription would have been missed if MSG transcripts were to be made in a manner that is independent of

Table 2 Unknowns Concerning MSG

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1. The number of MSG genes that can become attached to the UCS is high, but it is not clear that all MSG genes contribute to the diversity seen at the UCS.
 2. The frequency at which MSG genes move to the UCS.
 3. The mechanism by which MSG genes move to the UCS.
 4. MSR genes may recombine with MSG genes that are at the UCS. Hence, MSR genes could contribute to MSG diversity, even though MSR genes do attach themselves to the UCS.
 5. It is possible that MSG genes that are not attached to the UCS may be transcribed as mRNAs that lack the UCS. These mRNAs would lack the ATG codon used by UCS-containing mRNAs but might be translated from some other start codon. They would also lack the leader peptide provided by the UCS but may be able to move into the ER anyway.
 6. The putative UCS-MSG precursor protein has not been shown to function as such.
 7. The number of MSG protein isoforms expressed per organism has not been established.
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the UCS. Although the UCS appears to be needed for translation and transport to the surface, it is possible that these processes could occur without the UCS. (In entertaining the possibility of UCS-independent expression of MSGs, it is important to distinguish MSGs from MSRs, which are known to be expressed without being linked to the UCS.)

A major impediment to proving that any particular MSG gene is not expressed in a particular organism is the fact that the populations of organisms obtained from naturally infected rats tend to express many MSGs. This tendency means that lack of expression of a particular gene in some organisms in the population would be masked by expression of that gene in other organisms in the population. To ultimately either prove or disprove the antigenic-variation hypothesis, it will be necessary to produce clonally derived populations.

V. MSG in Other Species

All *Pneumocystis* species that have been studied via analysis of proteins on denaturing polyacrylamide gels exhibit a very abundant protein that migrates between 95 and 140 kDa. These species are *P. carinii*, and *P. wakefieldiae*, which are both found in rats, and *Pneumocystis* from ferrets, mice, rabbits, pigs, monkeys, and humans (2,3,5–16,20,21,27,90–94).

MSG genes have been shown to exist in six species of *Pneumocystis*: *P. carinii*, *P. wakefieldiae*, and *Pneumocystis* from ferrets, mice, monkeys, and humans (39,43,52,53,90,92,94–99).

The sequence of the UCS locus has been examined in *P. carinii* and *P. wakefieldiae* and in *Pneumocystis* from ferrets, mice, and humans (56,92,98–100). Each

species has its own UCS sequence. All known UCS sequences encode similar peptides. Nevertheless, the UCS locus of *P. wakefieldiae* appears to have evolved differently from that of *P. carinii*. The DNA encoding most of the *P. wakefieldiae* UCS is not unique in the genome. Instead, only the first 97 nucleotides are encoded at a unique locus. Sequences identical to the remainder are located on seven different chromosomes (98). These data show that one cannot infer details about all species of *Pneumocystis* from studies on one, and they serve to further illustrate how different the members of the genus can be.

VI. MSG as an Antigen

Host defenses can successfully attack *P. carinii*. The role of MSG in either allowing or preventing this vulnerability is not clear. Serum antibodies and T cells that recognize determinants on MSG occur in animals and humans (8,17–20,101–106). The response to MSG, however, has been notably variable. When human serum specimens have been screened for reactivity to MSG proteins by immunoblot, the typical result has been that only a fraction of specimens react with the band at 120 kDa, where MSG is found (8,17–19). The observation that not all serum specimens recognize MSG could be taken to mean that antibodies to MSG are absent from some individuals. However, this conclusion seems less than compelling in light of the realization that immunoblots may contain one MSG while a patient may have encountered another.

While it is clear that MSG can be recognized by the host immune system, the degree of protection such recognition confers is not understood. One way in which this issue has been explored is by using MSG to immunize laboratory animals that were subsequently infected with a *Pneumocystis*. In some cases, immunization conferred no protection (107). In other cases, there was a statistically significant reduction in the number of *Pneumocystis* organisms in the lungs of immunized animals (108–110).

The different outcomes of immunization experiments might be due to differences in methods, which include the following: (1) Different host species have been used in different studies; (2) different species of *Pneumocystis* have been used, both as the source of MSG, and as the challenge-organisms; and (3) the contents of the MSGs used could have differed because of two variables: (1) The organisms from which MSG was made came from animal lungs or (2) multiple MSGs isoforms were present in a single population, and different populations tend to express different sets of MSG genes. In some cases, the MSG used as immunogen was made in bacteria via expression of a cloned MSG gene, in which case only one isoform of MSG was present in the immunogen preparation. The variability of MSG could cause the response raised against an MSG immunogen not to recognize MSG determinants on challenge organisms. A similar phenomenon may be responsible for the lack of protection conferred by immunizing ani-

imals with one species of *Pneumocystis* and challenging them with another (111). Alternatively, native MSG (i.e., that prepared from *Pneumocystis* organisms) may not be an efficient immunogen because it is variable.

Another test of the role of MSG determinants in mediating immunity has been to administer monoclonal antibodies. In some cases, this treatment has been found to reduce infection burdens (12,112–117). However, natural antibodies can fail to protect against PcP. HIV patients and other immunocompromised hosts who develop PcP frequently have preexisting antibodies to the organism (17,118).

T-cell responsiveness to MSG and T cell–mediated protection have also been examined. The presence of protective T-cell epitopes on MSG and other specific *Pneumocystis* antigens has been shown by adoptive transfer and immunization experiments in some animal models (30,108–110,119) but not in others (107,120,121).

While there is no consensus on the role of MSG in eliciting a protective immune response, the behavior of MSG as an antigen and immunogen is not difficult to reconcile with the antigenic variation hypothesis, which posits that the MSG system is designed to allow an immune response to specific MSGs but to prevent or at least delay a protective response.

VII. MSG as a Factor in Adherence and Inflammation

Pneumocystis organisms bind to host cells and factors, and these interactions appear to both enhance growth of the microbe and provoke host defensive measures (see Refs. 122, 123, and 124 for reviews). MSG has been implicated in binding of *Pneumocystis* to type I pneumocytes and macrophages. MSG is known to bind to at least five host factors: fibronectin, vitronectin, surfactant protein D, surfactant protein A, and a mannose receptor on macrophages.

Fibronectin and vitronectin both accumulate in the lung during *Pneumocystis* infection. Beta glucan–enriched cell wall fractions from *P. carinii*, which presumably contain MSG, bind to these two integrins (125). Fibronectin binding to MSG appears to be involved in *P. carinii* attachment to lung cells (32,126,127).

Surfactant proteins are a major component of the alveolar exudates seen in PcP (128). Surfactant protein D (SP-D) is on the surface of *P. carinii* organisms when they are extracted from lung tissue. This binding is through saccharide-mediated interactions with MSG present on the surface of the organism (129). The number of carbohydrate recognition domains in SP-D is correlated with extent of binding to MSG (130). Surfactant protein A (SP-A) binds *P. carinii* in a specific and saturable manner. MSG has been implicated as a putative binding site for SP-A (131). Carbohydrate recognition domains on SP-A mediate the binding (132). Surfactants appear to be involved in reducing pathogenesis (133). The frequency and intensity of *Pneumocystis* infection were significantly greater in mice that lacked the SP-A gene, suggesting that surfactant may be a defensive

factor. Because these mice make no SP-A, it was possible to isolate *Pneumocystis* and alveolar macrophages free of this substance, allowing in vitro analysis of its effects. *Pneumocystis* organisms lacking SP-A did not bind efficiently to macrophage lacking SP-A. However, pretreatment of *Pneumocystis* with human SP-A resulted in a significant dose-dependent increase in adherence of *Pneumocystis* to macrophages. Thus, SP-A may be functioning as a nonimmune opsonin (133).

Pneumocystis organisms have been shown to bind to alveolar macrophages in vitro (134). Binding was inhibited by alpha-mannan, suggesting a role for MSG, which is known to be mannosylated. Also, soluble MSG competitively inhibited *P. carinii* attachment to alveolar macrophages, and MSG bound to alveolar macrophages in a saturable fashion. When macrophage membrane proteins capable of binding to MSG were isolated with an MSG-Sepharose column, a 165-kDa protein was detected. This protein was immunoprecipitated by polyclonal anti-mannose receptor antiserum (135). This protein may be a mannose receptor. The ability of the human macrophage mannose receptor to mediate binding of *P. carinii* was shown by engineering cultured monkey kidney cells to express this protein. Such cells gained the ability to both bind and ingest *P. carinii* (136).

Because MSG binds to macrophages via sugar residues on MSG, variation in MSG protein sequences might not have any effect on such binding. This possibility might seem antithetical to the hypothesis that MSG variation helps prevent the immune system from eliminating *Pneumocystis* organisms. However, *Pneumocystis* organisms were not efficiently taken up and killed by macrophages in the absence of antibody, suggesting a role for specific peptide determinants (134). Studies with mice defective for T-cell function also suggest a need for recognition of protein determinants (see Ref. 137 for review). For example, in the absence of CD4+ TCR alpha-beta cells, an activated phagocyte system was present, but not sufficient to cause clearance of *Pneumocystis* (138). In addition, MSG binding to macrophage receptors may work to the advantage of *Pneumocystis*. It has been reported that macrophages in vitro produced more soluble receptor in the presence of *Pneumocystis* organisms, and that *Pneumocystis* organisms with these receptor proteins on their surface were more resistant to macrophages. Soluble mannose receptor was detected in cell-free alveolar fluid from humans infected with HIV and *Pneumocystis*, but not in alveolar fluid from healthy controls. Soluble receptor was found in association with extracellular clumps of *Pneumocystis* in the lungs of mice with *Pneumocystis* pneumonia and was associated with *Pneumocystis* organisms purified from these mice. When purified *Pneumocystis* organisms were incubated with soluble receptor, they were phagocytosed less readily by alveolar macrophages than were control organisms. (139).

MSG has also been implicated in inducing inflammation via interactions with host cells (140,141). For example, incubation of an epithelial cell line with MSG increased IL-8 release (142).

VIII. Conclusion

The story of surface antigens of *Pneumocystis* is relatively well developed compared to other aspects of this ill-understood pathogen. Nevertheless, it would be surprising if many more antigens were not discovered in the future. Of the several surface antigens that are currently known, MSG is perhaps most intriguing, given its potential for variation.

The *P. carinii* genome carries numerous copies of ORFs (MSG genes) encoding different versions of a protein (MSG) known to be abundant and on the surface of the microbe. Hypothetically, this family of ORFs could confer the capacity to vary the surface, provided that different family members are expressed in different microbes. Switching the antigen on the surface could be accomplished by switching off expression of one ORF and switching on another. There is substantial evidence supporting this hypothesis, including 1) the identification of a locus that is associated with expressed MSG genes, 2) association of diverse MSG genes with this expression locus, and 3) correlation of expression of a surface epitope with the presence of the gene encoding it at the expression site. Direct evidence for variation is scarce but should be obtainable.

It seems probable that *Pneumocystis* uses surface variation for multiple purposes. Variation of surface antigens is a common phenomenon among microbes that colonize mammals. In some cases, variation has been shown to be vital to avoid death at the hands of the host immune system (76,143,144). In other cases, variation appears to be important for additional reasons (144–146).

List of Abbreviations

MSG: major surface glycoprotein

gpA: glycoprotein A

kDa: kilodaltons

Da: daltons

cDNA: complementary DNA

PRTI: protease 1

MSR: MSG-related

ORF: open reading frame

PFGE: pulsed field gel electrophoresis

UCS: upstream conserved sequence

mRNA: messenger RNA

Lambda clones: DNA fragments cloned by insertion into a vector made from the phage lambda

PCR: polymerase chain reaction

kb: kilobases

ER: Endoplasmic reticulum

C11 EES: C11 epitope encoding sequence

GPI: glycosylphosphatidylinositol

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5

Signal Transduction and Cellular Communication

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I. Introduction

Even simple unicellular fungi such as *Pneumocystis* have evolved sophisticated mechanisms to sense and respond to environmental cues by activating switches that result in coordinated changes in cellular physiology. The ability of the organism to respond is critical to survival within the host environment, for adaptation to host immune responses, and for regulation of life-cycle and cell-cycle events (1). These signaling pathways, in most instances, comprise protein kinases and phosphatases whose activity is regulated by cell surface or intracellular receptor proteins often in conjunction with GTP-binding proteins (Fig. 1) (2). A myriad of additional intracellular proteins interact with the core signaling pathways to regulate and coordinate function. Activated signaling proteins act directly on cellular target proteins modulating protein activity or on transcription factors regulating target protein expression. Although the components and interactions of these signal transduction pathways are well characterized in model yeasts, relatively few components have been identified in *Pneumocystis*. To date, all characterization of these components has been performed in rat-derived *Pneumocystis carinii*.

II. GTP-Binding Proteins

The study of signal transduction in *P. carinii* began with the discovery of a gene encoding a G-protein alpha (*pcg1*) (3). This gene was shown to exist in single

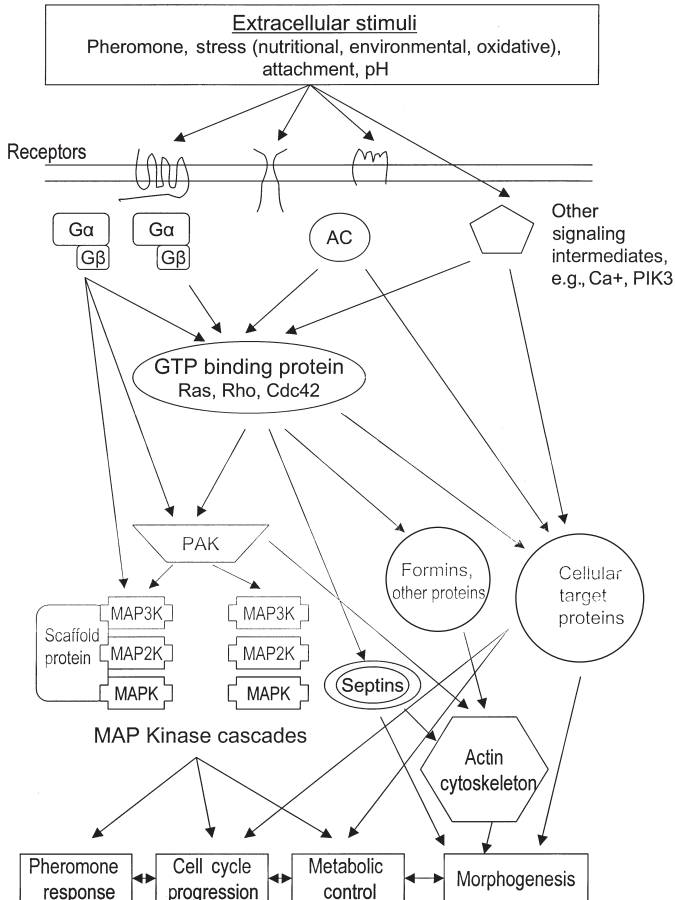


Figure 1 Signaling pathways activated by extracellular stimuli in most instances comprise cell surface or intracellular receptor proteins, often in conjunction with GTP-binding proteins, which regulate protein kinases and phosphatases. Additional intracellular proteins interact with the core signaling pathways to regulate and coordinate function.

copies in both the *P. carinii* and *P. wakefieldiae* (formerly known as *P. carinii* f. sp. *ratti*) genomes and to contain nine introns. The predicted polypeptide sequence of Pcg1 showed up to 53% identity with other fungal G- α proteins, such as GNA-2 from *Neurospora crassa*. Northern analysis demonstrated expression of *pcg1* in *P. carinii*, and some alterations in expression levels were observed in rich versus starvation medium conditions over time in the short-term maintenance

system. The 40-kDa Pcg1 protein was also shown to exist in extracts of *P. carinii* (3).

G- α proteins are highly conserved elements of signal transduction pathways, and the discovery of this protein was the first demonstration of the existence of signal transduction pathways in *P. carinii*. Although higher eukaryotes have been shown to possess many G- α proteins, there are far fewer examples of them in fungi (4). G- α proteins in fungi are involved in mating, pheromone response, and nutritional responses (5–7). G- α proteins in fungi can either interact with additional subunits of a heterotrimeric G protein or can act alone, as in the case of *S. pombe* (4,6). A gene encoding a G- β subunit in *P. carinii* has been isolated (accession number AF306565), but its role in signaling has not been determined. Therefore it still unknown whether interaction with other G-protein subunits is required for Pcg1 activity.

Sequence analysis of Pcg1 showed notable homology with other G- α proteins in the GTP binding regions and the existence of a consensus myristilation site at the amino terminus (3). Pcg1 does not appear to be a member of the G- α_i subclass but does contain the arginine residue necessary for cholera toxin ADP-ribosylation (3). The precise function of Pcg1 remains unknown; however, it closely resembles GNA-2 from *N. crassa* and CPG-1 from *Cryptonectria parasitica*. GNA-2 appears to have overlapping functions with GNA-1 in *N. crassa* (5). Deletion of *gna2* alone has no effect, but deletion of *gna2* in a $\Delta gna1$ strain accentuates the $\Delta gna1$ phenotypes. These phenotypes include a slower rate of hyphal apical extension on hyperosmotic media and female infertility (5). More recently, expression of a gene encoding a second G- α protein, Pcg2, in *P. carinii* was noted. Pcg2 has greatest homology with GNA-1 from *N. crassa*, but its role in *P. carinii* has not been determined (Smulian et al., unpublished data). CPG-1 in *C. parasitica* appears to be involved in regulating virulence, reproduction, and hydrophobin gene expression (6). These examples suggest that Pcg1 could be playing a role in such crucial processes as virulence and reproduction of *P. carinii*.

The comparison of the *P. carinii* and *P. wakefieldiae* copies of *pcg1* was an early example of justification for designating *P. carinii* and *P. wakefieldiae* as separate species. The homologues are only 82.5% identical at the nucleotide level and 92.1% identical at the amino acid level, differences that are greater than would be expected for mere strain differences (3,8).

III. Kinases

Phosphate transfer, the reaction catalyzed by all kinase, is a common step in many biological pathways. A subfamily of these kinases has elicited most interest because of their role in signal transduction and cellular regulation (9). This group, designated as protein kinases [eukaryotic protein kinases (ePK) and atypical pro-

tein kinases (aPK)], catalyze ATP-dependent phosphorylation of serine, threonine, and tyrosine residues on target proteins, changing their behavior in signaling pathways. Thus, protein phosphorylation is the most common on/off switch used in biology. While these protein kinases play a major role in signal transduction and regulation of many global biological functions, other kinases—such as the adenylate kinases, carbohydrate kinases, and deoxynucleoside kinases—are important components of specific pathways and regulators of pathway activity.

Examination of the yeast genome has identified 110 eukaryotic protein kinases that share a conserved catalytic domain (10). An additional 20 atypical protein kinases lack sequence homology but have structural similarity to ePKs. These kinases comprise ~2% of all genes within the yeast genome, encoding one of the largest protein families. A hierarchical classification of kinases has been developed, based primarily on kinase domain similarity, ordering kinases into groups, families, and subfamilies based on the taxonomy of Hanks and Hunter (11). Six major kinase groups are conserved, from yeast to metazoans, while an additional three—the tyrosine kinase, tyrosine kinase-like, and receptor guanylate cyclase groups—are found only among metazoan species (9). These groups are divided into 209 subfamilies, of which 51 are common to the kinomes of *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (worm), *Drosophila melanogaster* (fly), and *Homo sapiens* (human). Seven protein kinase subfamilies found in the yeast genome are not found in the worm, fly, or human kinomes but are conserved in *Schizosaccharomyces pombe*, mediating stress responses, cell wall signaling, cell cycle regulation, and small-molecule transport. An additional nine kinases of yeast have no close homologue in any species. This classification allows inference of functional similarities, including regulation of catalytic activity, substrate specificity and cellular localization. These inferences are especially important in *Pneumocystis*, where experimental determination of function is largely lacking and, when characterized, is mostly determined by expression and complementation in heterologous systems. Experimental characterization of some members of two groups of kinases has been performed, those involved in MAP kinase pathways and those involved in cell cycle regulation.

IV. Mitogen-Activated Protein Kinase Pathways

The first documentation of the involvement of MAP kinase pathways in *P. carinii* came with the discovery of a MAP kinase named PCM or Mkp2 (12,13). As with *pcg1*, *PCM/mkp2* was discovered by degenerate polymerase chain reaction (PCR) cloning using consensus kinase domain sequences. The *PCM/mkp2* gene, like *pcg1*, appears to exist on a single chromosome in the *P. carinii* genome. PCM/Mkp2 shares sequence homology with many MAP kinases, most notably those involved in growth regulation. PCM/Mkp2 was shown to be an active kinase by demonstrating the phosphorylation of MBP and histone H1 by material

immunoprecipitated with anti-Mkp2 antiserum (12). More recently, PCM/Mkp2 has been shown to be a functional kinase capable of complementing a *S. cerevisiae* *fus3Δ kss1Δ* mutant deficient in the pheromone or pseudohyphal filamentation pathway (14).

The *P. carinii* organisms used in initial immunoprecipitation experiments were untreated other than to the degree required to isolate the organisms from infected rat lungs (12). Therefore, it could be that PCM/Mkp2 is activated under normal growth conditions, suggesting a possible role in growth and proliferation of the organism. Subsequent studies demonstrated significantly greater PCM/Mkp2 activity in trophic forms of the organisms compared to the cystic forms (14). Levels of *PCM/mpk2* mRNA in trophic forms were found to be double those in cyst forms, suggesting both transcriptional and posttranslational regulation of PCM/Mkp2. The significant homology of PCM/Mkp2 to known pheromone-responsive MAP kinases and its activity in the pheromone or pseudohyphal filamentation pathway of *S. cerevisiae* suggest an involvement in the proliferation and differentiation of the organism (13–17). The HMG-containing transcription factor PcStell1 was shown to be one in vitro target of PCM/Mkp2 (18). PCM/Mkp2 is thought to be the MAP kinase involved in a form of mating pathway in *P. carinii*, and other putative members, such as Ste3, Ste20, of such a pathway have also been identified (19). As more members of this pathway are identified, the precise roles of the pathway and its components will be elucidated.

A second MAP kinase apparently involved in a different signaling pathway has also been identified. Similarity screening identified the *mkp1* gene, and its predicted amino acid sequence was shown to be homologous to SLT2 from *S. cerevisiae*, Mkc1 of *Candida albicans*, and Mps1 from *Magnaporthe grisea*, among others (13). Overexpression of Mkp1 could restore the growth rate and partially complement the elevated temperature lysis defect of a *S. cerevisiae* *slt2 Δ* mutant. Mkp1 was also activated in vitro in *P. carinii* upon exposure to oxidative stress (13).

Mkp1 is believed to be the terminal kinase of a pathway in *P. carinii*, analogous to the cell integrity pathway of *S. cerevisiae*. Evidence for this hypothesis is due not only to sequence homology to SLT2 and related proteins but also to its ability to effectively replace SLT2 in *S. cerevisiae* under conditions of stress (13). It is certainly intuitive that *P. carinii* would possess a cell integrity pathway, given that such pathways can protect the organism from stresses such as hypoosmolarity, oxidative stress, nutrient deprivation, and heat (2). Many or all of these conditions could exist within the lungs of mammals. Therefore the existence of a cell integrity pathway in *P. carinii* could certainly enhance its survival in the alveolus (13).

Lending further evidence for the involvement of Mkp1 in a cell integrity pathway is the finding that Mkp1 interacts with the *S. cerevisiae* transcription factor Rlm1 (20). In this study, further analysis of Mkp1 mutants led to a greater

understanding of the role of the residues within the activation loop of Mkp1 for kinase activity and substrate binding. It was determined that the first TEY motif (T182, Y184) is not necessary for complementation or kinase activity but may play a role in binding Rlm1. The second TEY motif (T186, Y188) is important for complementation, kinase activity, and Rlm1 binding. However, mutations in both motifs were insufficient in eliminating the ability of Mkp1 to complement an *slt2* Δ mutant and bind Rlm1. It appears that activation of Mkp1 is not required for binding to Rlm1, although it is unfortunate that an activated Mkp1 could not be included in the binding studies for the sake of comparison. The low level of complementation and Rlm1 binding observed in the Mkp1^{T182A, Y184F, T186A, Y188F} mutant could be an indication that other proteins may be involved in the activation of Rlm1 or that activation of Mkp1 may not be required for Rlm1-dependent transcription. Mkp1 may also have a function independent of its phosphorylation activity. ATP binding of Mkp1 appears to be more critical in Rlm1 binding than activation state. Mutation of the K52 residue eliminates not only the complementation and kinase activity but also its ability to bind Rlm1 (20).

Analysis of the expressed sequence tag library of *P. carinii* revealed the presence of another putative member of a cell integrity pathway named Mkk1 (Ferguson and Smulian, unpublished data). Mkk1 is highly homologous to MAP kinase kinases from fungal cell integrity pathways such as MKK1 from *S. cerevisiae* and both Skh1 and Wis1 from *S. pombe*. Southern analysis of the *mkk1* gene revealed its presence on a single chromosome in *P. carinii* and Northern analysis demonstrated expression of the gene. Initial complementation studies of *S. cerevisiae* and *S. pombe* MAPKK deletion mutants have shown the ability of *P. carinii* Mkk1 to serve as a cell integrity pathway MAPKK. Constitutively active mutants of *P. carinii* Mkk1 can partially complement the caffeine and vanadate resistance defects of the *mkk1* Δ /*mkk2* Δ mutant of *S. cerevisiae* and the sensitivity to caffeine and high salt seen in a *S. pombe* *skh1* Δ mutant (Ferguson and Smulian, unpublished data).

The putative MAP kinase kinase kinase (MEKK) component of this pathway, PcBCK1, has also been identified (21). Expressed in a *S. cerevisiae* *bck1* Δ mutant strain, PcBCK1 corrected the temperature-sensitive cell lysis defect. Expression of PcBCK1 allowed maintenance of the temperature-inducible beta-1-3-glucan synthetase gene *FKS2*. PcBCK1, Mkk1, and Mkp1 represent the best-characterized kinase cascade in *P. carinii* and should allow further characterization of interaction of cascade elements within the organism.

V. Cell Cycle-Related Kinases

Signal transduction pathways in fungi are involved in a variety of cellular processes, not the least of which have to do with the regulation of cell division. In a series of studies by Limper et al., the existence of three key proteins seemingly

involved in the regulation of cell division in *P. carinii* was demonstrated and possible roles were discussed. These proteins include a Cdc2 kinase homologue, its cognate Cdc13 B-type cyclin homologue, and a Cdc25 mitotic phosphatase homologue (22–24).

Cdc2 proteins are serine-threonine kinases that are normally essential for entry into mitosis and at the G1 restriction point (22,25,26). The *P. carinii* Cdc2 was discovered through the use of an antibody specific to the highly conserved PSTAIR amino acid sequence of Cdc2 proteins (22). A protein was immunoprecipitated with the anti-PSTAIR antibody that possessed kinase activity. By using techniques to enrich for either the trophic or cystic forms of *P. carinii*, it was determined that there was higher Cdc2-like activity in trophic forms than in cystic forms. Furthermore, when the *P. carinii cdc2* gene was introduced into a *S. pombe cdc2* temperature-sensitive mutant, growth at the nonpermissive temperature was restored (22). These studies indicated that *P. carinii* Cdc2 is a functional kinase both in vitro and in vivo.

The central role of cell-cycle control in the control of life cycle and replication implies a critical role of Cdc2 in these events in *P. carinii*. The observation that Cdc2-like activity is greater in trophic-form enriched preparations than in cystic-form preparations could be indicative that its activity is more important in mitosis than in meiosis. Cdc2 proteins are traditionally involved in entry into S phase from the G₁ phase and the transition from G₂ phase to M phase of the cell cycle, which may be consistent with greater activity in trophic *P. carinii*. Activity of Cdc2 proteins is regulated posttranslationally through interaction with other proteins and is regulated throughout the cell cycle (22,25). Although the precise role of Cdc2 in *P. carinii* is not yet known, it is likely that its function is analogous to that of other fungal Cdc2 proteins.

Further evidence for the role of Cdc2 in cell-cycle regulation in *P. carinii* was the discovery of its putative cognate B-type cyclin Cdc13 (23). Cdc13 in *S. pombe* is critical for Cdc2 activity, and it is likely that a Cdc13 protein would also play an important role in *P. carinii* (23,27). The *P. carinii* Cdc13 homologue was identified using antibodies specific for B-type cyclins. A protein complex was immunoprecipitated from *P. carinii* that possessed Cdc2-like kinase activity using the anti-B-type cyclin antibodies. The genomic and cDNA *cdc13* genes were cloned and sequenced. The *cdc13* gene, like other *P. carinii* signal transduction-related genes, is found on a single chromosome. A *P. carinii* Cdc 13-specific antibody was generated using a 15-amino acid synthetic peptide from the predicted amino acid sequence of Cdc13. Immunoprecipitation experiments yielded kinase-active apparent Cdc2/Cdc13 complexes. These complexes were variably active over the *P. carinii* life cycle, showing greater kinase activity in cystic form complexes than in trophic form complexes. *P. carinii* Cdc13 was also able to restore growth of a temperature-sensitive *cdc13* mutant of *S. pombe* at the nonpermissive temperature (23).

Typically, Cdc2 proteins must interact with their cognate B-type cyclins to achieve and maintain an active conformation (23,27). Therefore, given the existence of Cdc2 in *P. carinii*, it is not surprising that a Cdc13 would be present as well. It is assumed that the kinase activity shown in the anti-Cdc13 antibody-precipitated complexes was due to the presence of associating Cdc2 proteins, although unfortunately this was not directly demonstrated. It is somewhat surprising that the Cdc2/Cdc13 complexes show greater kinase activity in cysts than in the trophic forms, which is contradictory to the activity of anti-PSTAIR antibody precipitated Cdc2.

There are a number of possible explanations for the observation that complexes precipitated with either the anti-PSTAIR antibody or with an anti-Cdc 13 antibody show contradictory patterns of activity. The anti-PSTAIR antibody is not completely specific for Cdc2, given that the PSTAIR sequence is present in other kinases as well (24). Therefore the activity present in anti-PSTAIR-precipitated complexes could be from multiple kinases. It is also possible that, in *P. carinii*, Cdc2 may interact with different cyclins at different stages of its life cycle, or that Cdc13 could interact with a kinase other than Cdc2 that is highly active in cysts. Given that Cdc2 and Cdc13 from *P. carinii* can each functionally replace their respective homologues in *S. pombe*, it seems likely that they would function in an analogous manner in *P. carinii*, although further experimentation is necessary to define their precise roles.

Another protein known to interact with Cdc2 in *S. pombe* during the regulation of cell replication is the Cdc25 phosphatase (24,28,29). The *cdc25* gene from *P. carinii* was identified by degenerative PCR screening of *P. carinii* genomic DNA, followed by screening of a *P. carinii* cDNA library (24). The gene is present on a single chromosome, and the predicted amino acid sequence aligns well with other Cdc25 phosphatases. Heterologously expressed *P. carinii* Cdc25 was shown to have in vitro phosphatase activity. The $V_{\max}$ of the enzyme and its K_m for *p*-nitrophenol phosphate were measured and shown to be comparable to previously described Cdc25 enzymes (24).

The ability of *P. carinii* Cdc25 to complement the temperature-sensitive *cdc25-22* mutant of *S. pombe* varied with the different functions examined (24). Growth was restored at the nonpermissive temperature upon expression of *P. carinii* *cdc 25*; however, not all functions of the *S. pombe* Cdc25 were restored. Upon exposure to 200 Gy of radiation, the *P. carinii* Cdc25-complemented cells were able to restore the DNA damage checkpoint. However, when cells were treated with hydroxyurea, which inhibits ribonucleotide reductase, *P. carinii* Cdc25 could not restore the DNA replication checkpoint (24).

The *S. pombe* Cdc25 is a key regulator of cell-cycle progression, and its activity is regulated by the Chk1 and Cds1 kinases (24,30). The fact that *P. carinii* Cdc25 behaves differently at the two checkpoints indicates that a dissociation of checkpoint pathways at the Cdc25 level may exist. There could be distinct regula-

tory domains on Cdc25 proteins that differentially regulate the two checkpoint pathways (24). Given that *P. carinii* Cdc25 can substitute for the *S. pombe* protein to restore growth at elevated temperatures and the DNA damage checkpoint indicates that Cdc25 likely plays a key role in regulating progression through the life cycle.

VI. Receptor Proteins

Surface proteins of *Pneumocystis* have been examined largely as elicitors of a host immune response and as mediators of attachment to host cells (31–33). The most abundant surface protein, MSG, shares many genetic and biological aspects with adhesins of *Candida* and *Saccharomyces* and with variable surface proteins of organisms such as the trypanosomes and *Borrelia* (34,35). No available data suggest that MSG or other surface molecules such as the subtilisin-like proteases encoded by the PRT family of genes play a role in signaling, although this has not been directly examined.

Two putative receptors have been identified in *Pneumocystis*. The gene encoding a putative G protein–coupled **a**-factor pheromone receptor, *Ste3*, was discovered as part of the expressed sequence tag (EST) database. The *ste3* gene was cloned and the surrounding locus was also examined revealing the existence of genes encoding *Ste12*, a homeodomain transcriptional regulator; *Ste20*, a protein kinase; and a potential pheromone mating factor (19). This cluster of genes, a potential mating locus, provides supportive evidence for the existence of a sexual replication cycle in *P. carinii*. This is further supported by the expression of a putative pheromone response pathway MAP kinase encoding gene *PCM1mkp2*, (14) (Smulian et al., unpublished data), and other genes and transcripts encoding signaling components of a pheromone response pathway. In addition, transcripts of a number of genes which in other organisms are expressed solely during meiosis were identified in the EST database. However, in *Pneumocystis* these gene products could be used for other sensing functions.

The *ste3* gene contains two introns that can be alternatively spliced, leading to transcripts of different lengths and potentially to two different polypeptides (19). *Ste3* from *P. carinii* is homologous to other fungal **a**-factor pheromone receptors. There appears to be only one copy of *ste3* in the genome, which is located on the 680-kb chromosome I of *P. carinii* and on a 345-kb chromosome in *P. wakefieldiae* (19).

Other than a probable restriction polymorphism in *mts2* in the mating locus, there appears to be no evidence at this time of a second mating locus (19). *P. carinii* could be a true heterothallic organism and only mating type α cells were present in this study. It is also possible that both mating types could exist within the same lung and there could be disequilibria of mating types within the populations studied (19). It is likely that the completion of the *P. carinii* genome

sequencing project will provide the information needed to resolve the question of how sexual replication in *P. carinii* proceeds.

A second receptor protein has been reported in *P. carinii*. A cDNA, *ecmR*, isolated by hybridization screening of a *P. carinii* library was identified as encoding a putative extracellular matrix protein receptor, EcmR (36). The predicted protein showed high homology to the amino terminus of the murine and human laminin receptor proteins but little homology in the carboxyl third of the protein. Expressed recombinant EcmR protein appeared to bind laminin and fibronectin, although there appeared to be no competition between them for binding to the recombinant protein. While expression of the putative receptor protein was shown in *P. carinii* lysates, localization of expression was not performed. More recent BLAST analysis of the putative receptor sequence with protein databases suggests a greater degree of homology of EcmR to the 40S ribosomal protein S2. EcmR demonstrated 60% identity and 74% homology with the S2 orthologue of *Neurospora crassa* and *S. pombe*. Further studies will be necessary to determine whether *PecmR* encodes a S2 ribosomal subunit or an extracellular matrix protein receptor.

VII. Signal Transduction Components and the *Pneumocystis* Genome Project

The partial completion of the *P. carinii* genome sequencing project has led to the identification of many additional signal transduction components. The high degree of sequence conservation within the kinase domains allows identification of putative kinase genes based on sequence similarity analysis and designation within the kinase classification of Hunter and Hanks (11). This classification allows predictions of functional similarities, including regulation of catalytic activity, substrate specificity, and cellular localization. Thus far, 45 putative *P. carinii* kinases have been identified within all six kinase groups (Table 1). These kinases fall within 31 of the 62 kinase families identified in *S. cerevisiae*. One kinase, represented by the EST sequence s45b3, encodes a putative Ca/calmodulin-dependent protein kinase C. This class of kinases is not found in either *S. cerevisiae* or *S. pombe*, but orthologues have been identified in *N. crassa* and *Aspergillus nidulans*. The remaining putative kinases identified all have orthologues in *S. cerevisiae* or *S. pombe*. Additional signaling components identified thus far include genes encoding two G- β subunits, a14-3-3protein, eight small GTP-binding proteins, two GTPase activating proteins, and five phosphatase subunit genes.

VIII. Conclusion

Thus, although few signaling elements have been functionally characterized in *P. carinii*, the use of heterologous systems will allow characterization of the

Table 1 Putative Kinase of *Pneumocystis carinii*^a

Group	Family	<i>S. pombe</i> orthologue	<i>P. carinii</i> protein
AGC	PKA	Pka1	
	PKC	Pck 1/2	
	DBF2	SPAC24B11 (DBF20)	
	NDR	Orb6	
	MAST	Cek1	
	S6K	SPAC4G8.05 (Kin82)	
CMGC	GSK	Skp1	
	PRP4	Prp4	
	DYRK	Pom1	
	SRPK	Dsk1	
	CLK	Kns1	
	CDC2L	SPBC18H10.15	
	CDK9	P1014	
	PFTK	SPCC16C4.11 (Pho85)	
	CDC2	Cdc2	Cdc2
		Cdc7	
	MAPK	Spm1	Mkp2, PCM
		Spk1	Mkp1
CaMK	EMK	SPBC32C12.03 (Kin1)	
	SNF	Kin4	
		Gin4	
CKI	Yeast Unique	Mek1	
	CKI	Casein kinase I	
STE	MAP3K 1/2/3/5/6	Byr2	Bck1
	Ste20	Pak1/Shk1	Ste20
		Shk2/Pak2	Pak1
	MEK 1/2/5	Shk1	Mkk1
	NEK	SPAC19E9.02	
	Yeast unique	Cdc7	
		SPAC2C4.14C	
		SPCC320.12C (IplI)	
Other	IPL	– (Ca/Cal PKC)	
	CaMKK		
	RANI	Ran1	
	NRP/HAL5	Npr	
		Sat4	
	NRK	SPCC24A4.16	
Unclass	CKII	Casein kinase II	
		Nim1	

^a These are the assigned families of the 45 *P. carinii* kinases identified thus far in the genome. The *S. pombe* orthologue of each characterized and putative *P. carinii* kinase is listed.

multitude of new elements identified. Combined with standard biochemical analysis, molecular approaches will generate much new and useful information until such time as gene disruption and clonal population propagation of *P. carinii* is feasible.

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6

Transmission and Epidemiology

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I. Introduction

As the AIDS epidemic continues its progression through U.S. society, the at-risk populations have changed, and the incidence is now growing fastest among medically underserved, African-American, and female populations [1–3]. In these populations, *Pneumocystis pneumonia* (PcP) remains a leading opportunistic infection [4,5]. In countries considered to be “developing,” (i.e., Africa, Asia, the Philippines, and Central and South America), the number of those infected with HIV + has increased dramatically, sometimes affecting one-third of the population in some sub-Saharan countries. Although PcP rarely occurred in African adults in the first decade of the AIDS pandemic, the incidence has risen dramatically, as it has in other developing countries, reaching a 25 to 80% coinfection rate with tuberculosis and a mortality rate ranging from 20 to 80% [5]. Moreover, PcP is occurring more frequently in transplant recipients, especially in lung transplant patients [6] and those receiving high-dose corticosteroids for brain neoplasms, inflammatory or collagen-vascular disorders, and especially those with Wegener’s granulomatosis [7]. Trimethoprim-Sulfamethoxazole (TMP-SMX) remains the most efficacious prophylaxis and therapy for PcP [8,9]. Approximately 50% of *Pneumocystis jirovecii* isolates from HIV+ patients in different geo-

graphic areas contained a double mutation in the DHPS gene, which was associated with sulfa resistance in other pathogens [10,11]. These data suggest that resistance to TMP-SMX in human PcP may be emerging. A person-to-person spread is suspected as the primary mode of transmission for human PcP. However, basic concepts—such as the role of nonimmunosuppressed hosts as potential reservoirs of infection or the length of time organisms are carried and remain infective—are not known. Such information is of vital importance to the susceptible patient. Without adequate data for understanding the transmission cycles of PcP, recommendations for standards of care, such as isolation procedures for patients, are of little value. In the present chapter, the current knowledge regarding the transmission and epidemiology of *Pneumocystis* is discussed in the context of both human studies and animal model experiments.

II. General Life Cycle: What is Known and Unknown

The complete life cycle of all *Pneumocystis* species is poorly understood. The focus of most studies has been the replication phases within the lung of the immunosuppressed mammalian host [Fig. 1 (3)]. There, *Pneumocystis* appears

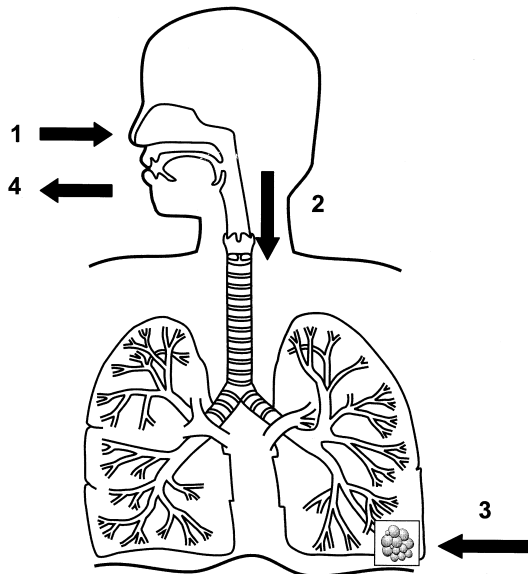


Figure 1 Unknown aspects of the *Pneumocystis* life cycle: (1) intake of infective propagules from the air; (2) route of travel from the upper respiratory tract to the lower; (3) replication phase within the lung; (4) mechanism of exit from the lung. (Picture adapted from Joanne Mikola Lesson Tutor at www.lessonstutor.com, with permission of author.)

to replicate asexually via binary fission (not budding), and sexually, likely mediated by mating types [12,13], resulting in an ascus containing eight ascospores. How the organisms exit from the lung is not known [Fig. 1 (4)], nor the process it uses to travel to the alveoli [Fig. 1 (2)]. It appears that the infection is acquired by an airborne route [14,15] [Fig. 1 (1)] (discussed below), but the agent of transmission has not been identified. In fact, the transmission and acquisition phases are almost completely unknown. Although recent studies have detected *Pneumocystis* DNA in neonatal human and rat populations, an assessment of potential reservoirs in the general population is only in its infancy. Significantly, there is no information about replication within the nonimmunocompromised mammalian lung, and little is known about its transmission or acquisition among intact hosts.

III. Mode of Transmission

In the late 1970s and early 1980s, Walzer and Hughes conducted a series of animal studies showing that *Pneumocystis* infection was likely transmitted by an airborne route [14,15]. *Pneumocystis*-free rats or mice could acquire the infection from infected animals housed in separate cages across the room or from animals housed within the same cage. Transmission through water, food, or cannibalism of infected lungs was not observed. Soulez et al. went on to use the rodent model of infection to determine that only a single day of exposure was the length of time necessary to pass the infection from infected mice with severe combined immune deficiency (SCID) to noninfected cage mates [16]. All of these studies clearly suggest that the agent of infection is contracted through the air, yet no laboratory has been able to identify a viable candidate for the infectious propagule. If one considers the dissemination of the tubercle bacillus as a potential model for transmission of *Pneumocystis*, then the infected hosts would serve to discharge the infective particle through coughing, wheezing, or other modes of dispersal from the lower to the upper respiratory tract and out into the external environment. Like droplet nuclei, these infective particles could stay suspended in the air for extended periods of time and take advantage of air or thermal currents for dispersion to other hosts.

Since the previous edition of this book in 1994, experimental evidence has been accumulating suggesting that the organism is contracted very early in life, probably through intimate contact, and that low numbers of organisms are able to initiate the infection. Human and animal studies support the concept that *Pneumocystis* organisms are able to exist and perhaps replicate at low levels in the lungs of nonimmunosuppressed hosts. Animal studies have also shown that most rats in commercial vendors are infected with *P. carinii*. To date, there is no evidence supporting an external amplification cycle that would provide a source of infective particles. The emerging picture of the transmission and life cycle of *Pneumocystis* from these studies and observations is one of an insidious and

highly infectious organism. In this chapter, we examine the advances in each of several aspects of transmission, from the origin of infection to its dissemination among susceptible hosts.

IV. Origin of Infection

The question as to where the infectious agent of pneumocystosis originates is one that has perplexed investigators from the time of the identification of pneumocystosis as an infectious disease. Whether the infectious propagules are generated from an *ex vivo* source in the environment or arise in the lungs of infected mammals and are exported to the air (or both) remains unknown to this day, almost 100 years after Chagas first observed the organisms. However, recent efforts to determine the reservoir of infection and other transmission-related events (e.g. length of carriage), using sophisticated molecular techniques like real-time polymerase chain reaction (PCR), suggests that the answers to these questions are close at hand.

A. Presence in Neonates

When do we first encounter these organisms? Serological studies performed in the 1970s through the 1980s showed that *P. jirovecii* was acquired early in life [17–21]. Most human beings became seropositive to *P. jirovecii* organisms or antigens by the ages of 2 to 4 years. More recent studies using the PCR targeting *P. jirovecii* genes (usually the mitochondrial large-subunit rRNA, mtLSU) support its early acquisition. Vargas et al. detected *P. jirovecii* DNA in nasopharyngeal aspirates in 24 of 72 infants (32%) suffering from mild respiratory infections [22]. Seroconversion developed in 67 of 79 (85%) of the same cohort of infants by 20 months of age. A recent case study reported by Miller and colleagues describe the apparent transmission of *P. jirovecii* from mother to infant using genotyping at three loci to support their contention [23].

The studies in neonatal infants provide strong evidence that contact with *P. jirovecii* occurs early, but the precise time of acquisition has not yet been determined. The study by Miller and colleagues raises the intriguing idea that the mother may be a source of infection. Using the tractable rat model of infection, my laboratory investigated the exact time of first exposure to the rat organism, *P. carinii*, using an oral swab–PCR technique. This technique uses the DNA extracted from swabs of the oral cavities of rats as template for amplification by the PCR with probes specific to *P. carinii* [24,25]. The presence of *P. carinii* in the oral cavities of rats was associated with the eventual development of *P. carinii* pneumonia after chronic immunosuppression [24], thus serving as a surrogate indicator of infection. Seven sets of rat dams and their pups were sampled for the presence of *P. carinii* by oral swab. The animals originated from a conven-

tional "open cage" room, housing rats of all ages known to harbor *Pneumocystis*. In addition, fetuses and maternal tissues were removed from three gravid females and tissue from two nongravid females as controls. All dams were positive and amplicons were produced from 80% [28 of 35] of pups within 2 hr of birth, from 97% [34 of 35] of the pups after 24 hr, and in all of the serially sampled pups by 48 hr (Fig. 2). No *P. carinii* amplicons were produced from 48 fetuses or their placentae taken by cesarean section. From these studies, we concluded that *P. carinii* was acquired almost immediately after birth and that placental transmission occurs rarely if ever in rats. The combination of clinical reports of *P. jirovecii* in neonatal humans and basic research studies in neonatal rats provide support for the hypothesis of neonatal reservoirs of infection and transmission.

B. Presence Within Adults

There has been a long-held belief that the pneumonia that occurred later in life was a reactivation of the initial infection, which had lain dormant for many years. Predicated upon this supposition, several studies were conducted over the previous four decades to search for the presence of *Pneumocystis* organisms in the lungs of human beings at postmortem examinations [26–30]. Histological detection of *Pneumocystis* in these diverse study populations ranged from 0 to 8%. In all studies, the presence of the organism was associated with an underlying immune defect. Organisms were not found in healthy adults. However, limitations in the design and conduct of these surveys make their interpretation difficult. In some cases, patients with underlying disease were selected for postmortem examination; review of medical records for diagnosis was often performed retrospectively; most importantly, the method of detection lacked sensitivity. Histological staining and subsequent microscopic screening of random lung samples for organisms that may be in low numbers is an insensitive survey method at best.

The search for the presence of *P. jirovecii* in the nonimmunocompromised human lungs continued to more recent times and employed more sophisticated sampling techniques. In one study, postmortem lung tissues from each of 15 nonimmunocompromised individuals were sampled in rotation from the right and left lower and upper lobes, homogenized, and the extracted DNA was used as template for *P. jirovecii*-targeted PCR [31]. In these and most other human studies, nested PCR using primers targeting the mitochondrial large subunit rRNA (mtLSU) of *P. jirovecii* has been the primary technique for detection. Although the limit of sensitivity of the PCR was estimated at 100-organism templates, none of the samples from the postmortem tissues yielded a positive result. This study implies that *P. jirovecii* may not be harbored for any significant length of time in the healthy individual. However, the method of sampling could again account for the lack of detection of organisms in small numbers residing in scattered foci.

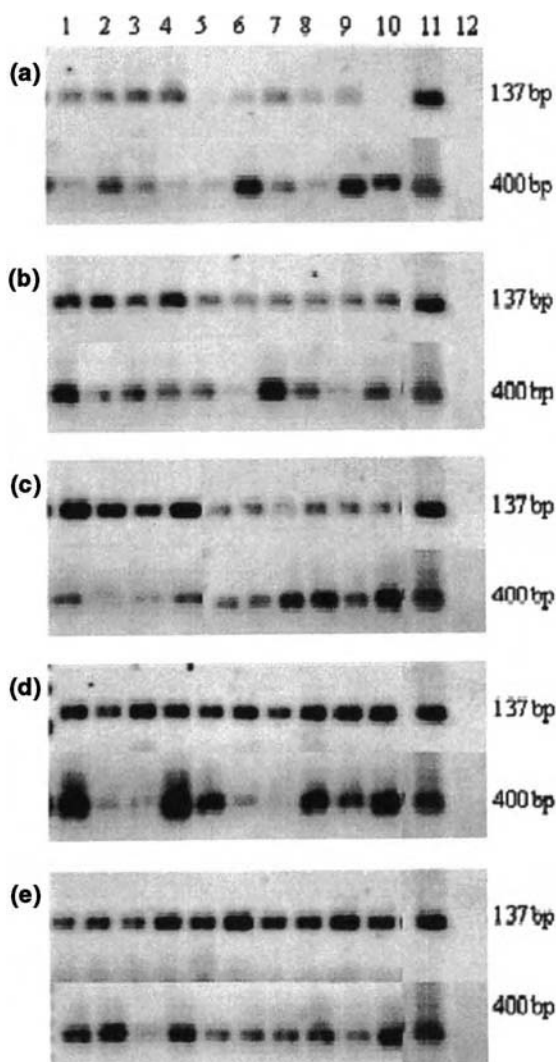


Figure 2 Oral swab samples taken at time points ranging from 1 to 2 hr to 1 week after pup birth. A. Oral swab samples collected from individual rats within 2 hr after pup birth; B. Oral swab samples collected 24 hr after birth from the same rats shown in A. C. Oral swab samples collected 48 hr after birth. D. Oral swab samples collected 72 hr after birth. E. Oral swab samples collected 1 week after birth. All panels show amplicons for Rcc (137bp) and rat globin primer sets (400 bp). For all panels, lane 1 represents oral swabs collected from the dam, while lanes 2 to 10 represent individual rat pup oral swabs; lane 11 is the positive control (*P. carinii* DNA, contaminated with rat DNA); lane 12 is negative control (water) (From Ref. 25.)

DNA extracted from bronchoalveolar lavage fluids, sputa, and oropharyngeal washes have been used as templates for amplification of *P. jirovecii*-specific genes and genetic regions as a means of determining infection and colonization. In an early study of sputum samples from asymptomatic patients, immunosuppressed patients, and heart/lung transplant patients, all 30 patient samples were negative, using an immunofluorescent method based on microscopic detection of organisms [32]. In contrast, 3 renal and 2 heart/lung transplant patients were positive by PCR. One of these 5 patients later developed symptomatic *Pneumocystis* pneumonia 6 weeks after the time of sampling. The authors of the study hypothesized that colonization likely occurred in the transplant patients due to the immunosuppressive effects of the antirejection therapy, but this colonization did not necessarily result in progression to fulminant infection, since 4 of the 5 patients did not develop *P. jirovecii* pneumonia.

Probable asymptomatic carriage has been shown for both healthy and HIV-infected individuals. In a recently published study, 47 samples of bronchoalveolar lavage (BAL) fluid were obtained from 16 HIV-positive individuals over a study period from 1990 to 2001 [33]. Nested or single-round PCR amplification targeting the small subunit and large subunit mtLSU showed that 35 of the 47 samples contained *P. jirovecii* DNA. Importantly, 18 of the positive samples came from patients who did not have clinical evidence of *P. jirovecii* pneumonia, suggesting that asymptomatic carriage occurred in these patients. Seven patients who did have an episode of *P. jirovecii* pneumonia were found to have asymptomatic carriage for up to 9.5 months after apparent resolution. Genotyping of these organisms by sequence analysis of four genetic loci revealed that the profile changed from that of the original infecting organisms. The combination of the change in genotype and the lack of detection of *P. jirovecii* DNA in 12 of the 47 samples are contrary to the theory that infections are derived from organisms acquired at birth.

In a larger study, 17 of 93 (18%) consecutive BAL fluid samples from HIV-negative patients undergoing diagnostic bronchoscopy were found to be positive for the presence of *P. jirovecii* by PCR [34]. Analysis of potential risk factors showed only glucocorticoid use (and not lung cancer or chronic obstructive pulmonary disease), was associated with the presence of *P. jirovecii*. Of 18 patients (44%) receiving the therapy, 8 produced *P. jirovecii* amplicons from their samples, while only 9 of 75 patients (12%) who did not receive glucocorticoids were positive.

Pregnancy may also be a risk factor for asymptomatic carriage. A recent study of 33 pregnant healthy women in their third trimester vs. 28 healthy women within 15 days of their menstrual cycle showed the presence of *P. jirovecii* DNA in 5 (15.5%) of deep nasal swabs from the pregnant women as compared to none in the nonpregnant women [35]. Evidence for the early acquisition of *Pneumocystis* in neonates with the mother as a potential source of organisms is accumulat-

ing, with evidence reported both in human [23] and animal studies [25]. Mother-offspring transmission is an important consideration for the management of infants born to HIV-infected mothers, who currently receive empiric anti-*Pneumocystis* chemotherapy at 1 month of age as their only prophylactic option [35].

Other factors appear to favor colonization with *P. jirovecii*. In a large study of 367 patients with suspected bacterial pneumonia, carriage of *P. jirovecii* (detected by the same PCR methods discussed above) was statistically associated with old age, concurrent disease, and steroid treatment [36].

A recent study conducted in Japan compared the presence of *P. jirovecii* in BAL fluid (determined by staining and PCR to the 5S rRNA gene) in 26 HIV-infected, 45 non-HIV immunosuppressed, and 10 immunocompetent patients with primary pulmonary disease [37]. None of the immunocompetent patients were positive by either method, and more HIV+ patients were positive by either method than the HIV- patients. None of the patients with a positive test developed PcP during the follow-up period, underscoring another question that must be addressed: What does the presence of a positive PCR or a few stained organisms mean in terms of disease development? There are no longitudinal studies to determine the length of carriage or its potential for transmission or ability to cause infection. There are limits to such studies in human beings, and animal models become necessary to begin investigation of these critical parameters.

C. Length of Potential Carriage

As a prelude to identifying the potential reservoirs of infection within a population, it is important to assess the members who are carrying the infection and then the length of time the organisms can reside in its host. We tested the length of time the *P. carinii* could reside in the healthy host and maintain its ability to cause infection by isolating the rats from colonies known to harbor *P. carinii* upon receipt, under barrier conditions and provoking the infection after 1, 2, 3, and 4 weeks of isolation. Pneumonia developed in rats kept under barrier for 4 weeks prior to immunosuppression, suggesting that the organism can be carried by healthy rats for at least this length of time [38–40].

Vargas et al. evaluated the length of time *P. carinii* could persist in the lungs of rats after fulminant infection [41]. After recovery from the infection by tapering of steroids, groups of rats were moved to barrier cages and reimmunosuppressed to provoke any latent organisms to cause disease. The presence of the organism was assessed by microscopic methods and PCR directed to the mtLSU. About 75% of the rats did not produce infection 1 year after their primary bout of PcP, causing the authors to conclude that the organism was cleared from these rat lungs. The average lifetime of a rat is about 2 years. Putting the persistence of organisms into this context, it should be appreciated that “clearance” of the organisms took about half of their lifetime, suggesting a rather lengthy persis-

tence. In addition, a full 25% of the rats did not “clear” the infection, further supporting the ability of *P. carinii* to persist in the immune-competent host lung. Furthermore, it should be noted that immunosuppression of older rats is often inefficient due to increased body weight. Failure to provoke the infection in some recovered rats may have been due to a partially debilitated but not sufficiently depleted immune system. The ability of these rats to transmit the infection was not addressed.

The data from these studies as well as those from the transmission experiments discussed below suggest that the organism may not reside indefinitely in a host but can remain for significant lengths of time. If the organism can then be transmitted during the time of carriage, even intermittently, it would appear that there is likely a constant circulation of *Pneumocystis* within the general populations of humans and rodents.

V. Transmission

A. Transmission Among Immune-Competent Hosts

The widespread prevalence of *Pneumocystis* in commercial rat colonies—as shown by PCR, serology and subsequent immunosuppression [24,40,42]—not only supports the very efficient dissemination of the infection throughout the members of a colony but also shows that the organism thrives and is transmitted among immune-competent hosts. Moreover, rats from such colonies develop fulminant PcP after chronic immunosuppression under barrier isolation, illustrating the infectivity of the organisms.

Recent results from studies evaluating the transmission of *P. murina*, the organism that infects mice, showed that immune-competent mice could transmit the infection to other immune-competent mice [43]. In these studies, the immune-competent mice were first housed together with SCID mice infected with *P. murina* for 3 weeks. Positive serology using an enzyme-linked immunosorbent assay (ELISA) technique served to document successful infection as well as their ability to transmit a viable infection to both immune-compromised and immune-competent *Pneumocystis*-naïve mice.

The pervasive presence of *P. carinii* among normal, healthy rats in commercial colonies and the experimental transmission of the infection among immune-competent mice strongly support the role of healthy hosts as potential reservoirs of the infection in a general population.

B. Transmission Among Immune-Incompetent Hosts

It is well known that *P. carinii* is transmitted from an infected immunosuppressed rat to an uninfected immunosuppressed rat. This mechanism is the basis for the “seeding” approach taken to establish *Pneumocystis* in a rat or mouse colony,

whereby an infected animal is placed in a cage with one or more animals for varying amounts of time to transmit the infection [44]. Within human populations, this issue becomes less clear, due to problems in quantifying past exposures to persons with PcP. Some of the less ambiguous data comes from very early reports of epidemic outbreaks of “interstitial plasma cell pneumonia” among orphan children in institutionalized settings after World War II [17,45,46]. This pneumonia was later identified as PcP by histological methods. The children, made immune incompetent by malnourishment and disease and being in close contact due to severe crowding, acquire the infection as it was communicated throughout their population. Several reports of cluster cases of PcP in adult human populations rendered immune incompetent by HIV or transplant chemotherapy are highly suggestive of transmission among susceptible populations (see Ref. 17 for review). For example, during a 22-month period, 5 renal transplant patients developed PcP apparently as a result of exposure from use of the same outpatient clinic waiting room as HIV patients [47]. These studies were indicative of transmission but lacked genotyping information that would have provided concrete evidence for epidemiological tracking. With the advent of molecular typing methods, support for direct transmission in some but not all cluster cases was provided. Most of these cases involved small numbers of patients, which could then be more completely assessed for potential exposures, but unfortunately they did not provide adequate numbers for strong statistical analysis. A very recent case-control study employing large numbers of HIV-infected persons with and without PcP (> 200 in each group) and a history of exposure to persons with PcP reported a lack of association between past exposures to persons with PcP and an increased odds for development of PcP [48]. However, as the authors pointed out, difficulty in quantifying past exposures and the length of exposure time are limitations of this research. Thus, transmission among immune-incompetent humans likely occurs, but many unknown factors influence the process.

C. Transmission from Immune-Incompetent *Pneumocystis*-Infected Hosts to Immune-Competent Hosts

Emerging evidence indicates *Pneumocystis* can be transmitted from immunologically debilitated and infected hosts to uninfected, immunologically competent hosts and subsequently to other individuals. For the first part of this sequence, Vargas et al. showed the potential for transmission from a *P. jirovecii*-infected host to noninfected individuals by tracing the appearance of *P. jirovecii*-specific amplicons in health care workers and relatives that had close contact with a child diagnosed with PcP [49]. Similarly, Dumoulin et al. showed immune-competent BALB/c mice exposed to *Pneumocystis*-infected mice produced *Pneumocystis*-specific amplicons (targeting the mtLSU) from the lungs after only 1 day of exposure but required 40 days of cohabitation to produce subclinical numbers of

organisms visualized by microscopic methods [50]. The question then becomes whether these colonized immune-competent hosts can transmit the infection.

D. Transmission from Immune-Competent Infected Hosts to Immune-Incompetent Hosts

Dumoulin and coworkers then investigated the potential for transmission from a transiently colonized immune-competent host to susceptible recipients [50]. *Pneumocystis*-specific amplicons were found in 9 of 9 recipient SCID mice after 40 days of close contact (cocaging) with immune-competent BALB/c carrier mice. Low numbers of cysts could be visualized only in the SCID recipients that were exposed to carrier mice that had the longest (20 day) exposure to *Pneumocystis*-infected mice (the first part of the experiment, described above). Thus, these investigators demonstrated that immune-competent mice exposed to a source of *Pneumocystis* infection could become at least transiently infected and then, in turn, transmit organisms to immune-incompetent recipients. Although the presence of *Pneumocystis* was apparent in the recipient mice, the ability to cause overt disease in these susceptible mice was not evaluated by these investigators. More recently, An et al. [51] were able to show that immune-competent BALB/c mice exposed to *Pneumocystis*-infected SCID mice were able to contract the infection and transmit it to other immune competent mice, but also to *Pneumocystis*-naïve SCID mice. Moreover, the second group of healthy mice that were exposed to the initial group of infected immune-competent mice to test transmission of the infection through normal healthy hosts were able to transmit the infection to the susceptible, immune-incompetent SCID mice during a 6 week period of cohousing.

VI. Ability to Survive in the Environment

The ability to survive in the environment is an important factor in the transmission of disease for many microbes. Such mechanisms include resistance to heat, drying, ultraviolet light, and chemical agents; the ability to compete with other microorganisms; and the ability to multiply in the environment or within a vector.

Few studies have assessed the ability of *Pneumocystis* to survive in the environment. Those that are available have been severely hindered by a lack of knowledge of the transmitting form and the inability to evaluate organism viability. Using organisms from lung homogenates, Ito et al. [52] reported that *Pneumocystis* from mice were unable to survive for long periods of time outside the mammalian lung and that most chemical disinfectants were able to kill the organisms, as assessed by their inability to infect SCID mice. The organisms also appeared to be very sensitive to high temperatures and drying. The only organisms able to cause infection were those subjected to freezing and thawing or stored

at -80°C . In contrast, Kaneshiro and Maiorano [53] found that putative organisms on cage filters and HEPA filters from blowers in infected rat rooms could remain infective to immunosuppressed rats if stored dry for up to 5 months at room temperature. It is difficult to reconcile these studies at this time because of many technical problem associated with the study designs. Ito et al. used organisms directly from the lung, which are not likely to represent the transmissive form. Kaneshiro and Maiorano exposed rats to objects captured on filters, which may or may not have been *Pneumocystis*. Both studies used inoculation of immunosuppressed animals as the test of viability. It is now well known that many rats from commercial vendors harbor latent *Pneumocystis* infections, which would confound interpretation of such viability assessments.

Detection of the infectious propagule of *Pneumocystis* infection remains elusive. In 1999, putative transmissive forms were presented by Chin et al. [54] that originated from air circulation filters, microisolator cage-top filters used to house *P. carinii*-infected rats, and from the tracheal tissue of infected rats. Analysis methods included light microscopic examination supplemented with histological stains and immunofluorescence. PCR was used to verify the identity of organisms placed in culture as *P. carinii*. It was hypothesized that there were two environmental forms of the organism that were about $6\text{ }\mu\text{m}$ in diameter, one that has a single nucleus and the other that is multinucleate. These findings have not yet been verified by another lab but remain as potential candidates of infection.

Icenhour and Cushion sampled animal colony room walls, floors, air ducts, and other surfaces by extraction of DNA from swabs taken from the different areas for the presence of *Pneumocystis* DNA using PCR directed to the mtLSU and three other nuclear genes [55]. All surfaces were positive for *P. carinii* DNA in the animal room and sequences of the amplicons were those of *P. carinii*. Collection of material from the swabs and reaction with magnetic beads coated with monoclonal antibodies directed to the surface antigen family of *P. carinii* resulted in positively staining forms, suggesting that both *P. carinii* DNA and surface structures were present. As a control, a Veterans Affairs hospital room with negative pressure used for tuberculosis isolation was sampled. PCR directed to rat *Pneumocystis* was negative, but PCR directed to the human *P. jirovecii* mtLSU was positive. The morphology of the particles collected from the environment of the animal room were not consistent with fungal spores; thus, these findings also await confirmation and additional investigation. Although the transmissive agent of *Pneumocystis* infection has not yet been conclusively identified, but the presence of organism specific DNA detected by the studies suggest that *Pneumocystis* may be abundant in rooms in which infected hosts reside.

VII. Infectivity

There is strong evidence that the transmission of *P. carinii* is very efficient and that few organisms are required for infection. Studies in our laboratories revealed

that rats with intact immune systems obtained from commercial rat vendors and maintained under barrier for up to 4 weeks prior to immunosuppression could still produce a fulminate infection after the standard 8 to 12 weeks of immunosuppression [38–40]. Microscopic analysis of histochemically stained whole lung homogenates of sentinel animals (nonimmunosuppressed rats) from these rat groups showed no *P. carinii* organisms, suggesting there were fewer than 10^4 organisms in their lungs, the threshold of detection. In one limited study [50], exposure of only a single day to a *Pneumocystis*-infected mouse was necessary to pass the infection to a *Pneumocystis*-naïve SCID mouse. Air sampling studies of patient room air, where the organism would be expected [56], and orchard air [57], with no obvious source, detected the presence of *Pneumocystis* DNA by PCR, but in very low levels. Neither study could identify any *Pneumocystis* organisms in the stained samples, suggesting that very low numbers of organisms were present.

Although the intratracheal inoculation model of PcP has been used for several years, no studies describing the outcome of different inocula levels on infection production have been published. We conducted four experiments to evaluate the ability of low numbers of *P. carinii* to produce infection [58,59]. These experiments were performed to aid in the understanding of the transmission of the infection, to examine the kinetics of the expression of the major surface glycoprotein genes (*MSG*), and provide a model of infection more closely simulating what we believe is the “natural” model in which low numbers of organisms initiate the infection.

Inoculation of dilutions of organism suspensions with 10, 10,000, and 10,000,000 nuclei into immunosuppressed rats produced heavily infected animals with averages of 2×10^8 , 3×10^8 , and 1×10^9 organisms respectively after 8 to 12 weeks of immunosuppression. The average number of *P. carinii* was only weakly related to dose, suggesting that as few as 10 organisms can initiate infections that give rise to rats with burdens equivalent to those inoculated with a million more organisms. Organism populations with decreasing complexity of the *MSG* genes at the expression locus were related to the decreasing inocula dosage [59]. Rats inoculated with 10 organisms produced *P. carinii* populations in which a single *MSG* sequence was at the expression locus in 90% of the organisms. By contrast, the inoculum used in this experiment contained at least 27 different *MSG* genes at this locus, suggesting that a severe bottleneck occurred. The presence of the same simple *MSG* profiles in cage mates suggests that in some cases, one rat may have infected the other in the cage. Even though both were inoculated, it is possible with such low dilutions that one of the rats did not receive any organisms. This transmission scenario is supported by the large difference in burden (20 million vs. 200 million) in two such rats, 271 and 272 [59].

The ability to initiate infection with as few organisms as ten nuclei or one theoretical cyst (which has eight nuclei) indicates that *P. carinii* is very efficient in its transmission and is a highly infectious organism. This was subsequently verified by detection of *P. carinii* in many rat colonies surveyed, including barrier facilities [24,42,60].

VIII. Influence of Environmental Factors on Transmission

Since *Pneumocystis* appears to use an airborne route of transmission as one of its methods of dissemination, it is plausible that external factors such as relative humidity or temperature might affect the prevalence of PcP infections. Surprisingly, very few studies addressing the role of weather or seasonal variation of PcP have been conducted. Those that have offer retrospective analyses with conflicting but intriguing results. In 1974, Walzer and colleagues found no association with the season in 194 confirmed PcP cases [61]. Miller et al., in 1992 looked at rainfall and temperature for a correlation with PcP in HIV+ patients and suggested that relative humidity and temperature may influence the infection, but they provided few data in support of this contention [62]. Hoover, in 1996 [63], suggested that colder temperatures may positively influence the spread of the infection, perhaps due to person-to-person transmission during colder seasons or owing to more favorable conditions for the transmissible form, since a higher incidence of PcP was observed during the first 6 months of the year in the United States and in northern regions in Europe [64,65]. Colder temperatures were also associated with a higher incidence of upper respiratory infections, which may have predisposed the hosts to PcP.

In our rat facilities, we observed that mixed infections of *P. wakefieldiae* and *P. carinii* increased in relation to single *P. carinii* infections at certain times during the year [66–68]. Since a seasonal association of the occurrence of human PcP has been inferred by some studies, we conducted a prospective study to determine the potential effects of environmental factors on the infections caused by these two species of rat *Pneumocystis*. A conventionally housed rat colony at the Cincinnati VAMC VMU was monitored for temperature, relative humidity, and rat census over a 2-year period. The room contained breeding colonies of Brown Norway and Long Evans rats obtained from Charles River in 1994. Rats were housed in shoebox or hanging rack cages open to the room air. The total rat census within the study room at a given time ranged from 70 to 200 rats during the study period. Immunosuppressed rats accounted for about 40 to 50% of the total, the rest being nonimmunosuppressed weanlings and breeders. A multiple logistic regression approach was chosen to model the environmental data. All variables were run independent of one another for analysis of effect and then combined. Based on data from over 600 rats, we found that the probability for a rat to have an infection consisting of only *P. carinii* increased as the relative

humidity increased ($p = 0.008$, slope = 0.0211), while the probability of *P. carinii*-only infections were associated with a small temperature range of 68 to 69°F ($p = 0.02$; slope = -0.1472). A higher rat census was associated with less of a probability of *P. carinii*-only infections ($p = 0.04$; slope = -0.008) while a heavier organism burden had a higher probability of being *P. carinii*-only ($p = < 0.0001$, slope = 0.366). The penultimate observation is consistent with other microbial systems in which a less robust species in competition with a stronger one can increase in number if the available host population expands, providing more resources [69]. As the available hosts decrease, the more aggressive species dominates, often pushing the less robust species to extinction.

IX. Host Factors

The success and extent of the severity of infection relies not only on the intrinsic factors of the agent but also on the characteristics of the host. Potential interactions between the agent and host include colonization, subclinical disease, and overt infection [70]. Host factors influencing the development of disease include the age at infection, immune status, comorbid conditions and diseases, and extent of invasive procedures, for example. The presence and severity of the host factors combined with the intrinsic virulence of the agent will shift the overlap of the host and agent and influence the areas of colonization as well as covert and overt infection. In the case of PcP, the balance shifts towards overt infection as the host becomes immunosuppressed, with the severity of infection increasing as immune function decreases.

The immunosuppressive state of the host predisposing to development of PcP has been studied at length and is not the focus of this chapter. What remains unclear is the role of the infected-*immunosuppressed* host in the transmission of infection. Equally unclear are the kinetics of colonization and covert *Pneumocystis* infection in the *nonimmunosuppressed* host. As discussed above, strong evidence for the presence of *Pneumocystis* in immune-intact hosts and neonates is accumulating. It then becomes essential to understand these early processes as a means towards control and prevention of *Pneumocystis* infection.

X. Real Time PCR: A New Tool to Determine Length of Carriage, Detect Transmission, and Progression of Infection

The antemortem tracking of the carriage, transmission and organism burdens in *Pneumocystis*-infected hosts has not been possible because no suitable long-term ex vivo cultivation method has been identified for any species of *Pneumocystis*. Recognizing this impediment, our laboratories explored the ability to track the progression of *P. carinii* infection in immunosuppressed rats using DNA extracted from oral swabs (described above) with real-time PCR [71]. Thirty-two male rats

were obtained from the same open cage rat colony at the Cincinnati VAMC VMU used for the early acquisition studies presented above. Rats were removed from the room and caged individually under barrier conditions to prevent transmission of infection that might occur between cage mates. Groups of eight rats were screened for the presence and levels of *P. carinii* in their oral cavities by real-time PCR at the beginning of the experiment and 8 rats each at 4, 7, and 10 weeks postimmunosuppression. At the same time points, the organism burdens of the lungs from each rat were quantified by microscopic enumeration of organism nuclei.

Linear regression was used to assess the correlation of the averages of the C_T s from each time point vs organism burden (Fig. 3). Thresholds were set at 41 cycles for the RT PCR and 10,000 nuclei for the microscopic detection. The lung burden is expressed on the Y axis as the log nuclei count per lung and the threshold cycles on the X axis. The lower the threshold cycle, the more *P. carinii* template was present. Thus, at 10 weeks, the organism burden was log 9, while the average C_T was 30. At lower burdens—e.g., 4 weeks, log 5—the C_T s ranged from 38 to 39. By extrapolation, an oral swab with a 36 C_T would predict a burden of $\sim \log 6.3$.

To determine whether the oral swab/RT PCR technique was sensitive to decreases in lung burden, a pilot treatment study was conducted using the same methodology described above. Two groups of rats were swabbed upon receipt into the study and at death. The C_T s and the organism burdens were determined from each lung homogenate at termination. Group 1, nontreated (4 rats) were

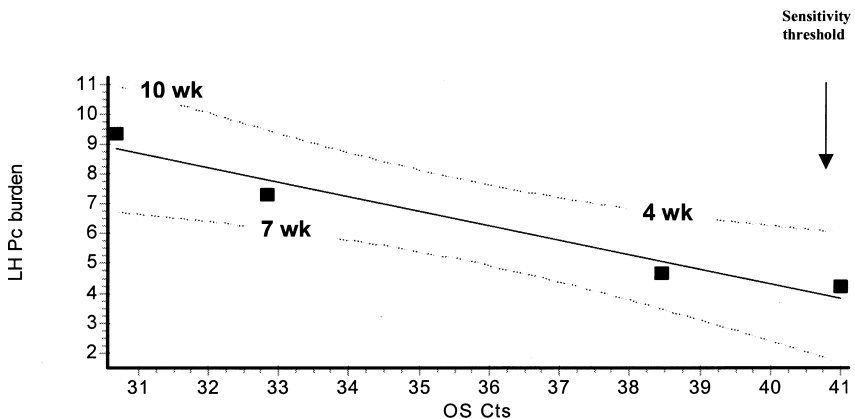


Figure 3 Correlation of RT PCR with *P. carinii* burden. The averages of the oral swab threshold cycles at each time point were plotted against the log counts of *Pneumocystis* nuclei. $R^2 = 0.9540$.

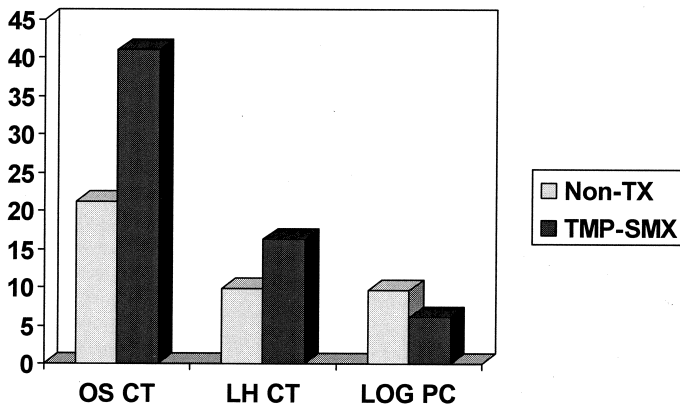


Figure 4 RT-PCR of untreated vs. TMP-SMX treated rats. OS = C_T from oral swab at death; LH = C_T from postmortem lung tissue; Log Pc = log of total Pc nuclei/lung. All nontreatment-treatment pairs were significantly different from one another ($p < 0.05$).

immunosuppressed for the 8-week period and group 2, treated (3 rats), received TMP-SMX (50/250 mg/kg/day PO) for 15 days prior to termination at 8 weeks. The data for the termination time point are shown in Fig. 4. The lower the C_T value, the greater the template abundance; thus, the oral swabs from nontreated rats had far more *P. carinii* templates in their oral cavities (average 21.2 ± 0.76 SEM) than the TMP-SMX-treated rats at death (41 ± 0). In fact, none of the treated rats had detectable targets as reflected by the score of 41, the threshold level of this technique. This difference was observed in the template abundance of the lung homogenate, (9.95 ± 1.0) for the non-treated rats vs. (16.3 ± 0.8) for the treated rats. These results were inversely proportional to the log Pc nuclei lung burden: (9.6 ± 0.3) for the nontreated rats and (6.17 ± 0.1) for the TMP-SMX-treated rats. As in the previous study, the RT PCR results of both the OS and LH reflected the organism burdens in the rat groups.

Thus, the oral swab-treat time PCR method can now be used as a surrogate method to assess whether the infection was transmitted to a recipient host, the length of carriage within an individual host, and the progression of the infection after initial contact.

XI. Conclusions

Although the complete life cycle—including the transmission, potential reservoirs, and infectious agent—have not been elucidated for any species of *Pneumocystis*, clinical and animal model studies are beginning to provide integral pieces to the puzzle of the natural history of this enigmatic infection. The sum of the

available studies indicate that (1) transmission likely occurs among immune-competent hosts, between immune-competent and incompetent hosts, and among immune incompetent populations; (2) neonates, immune-competent and immune-incompetent hosts may function as animate reservoirs, but there is no evidence to support inanimate reservoirs; (3) carriage of *Pneumocystis* may be lengthy; and (4) transmission appears to be very efficient. These observations have been gathered from several studies that do not fully define any of the parameters influencing these apparent transmissions, and more comprehensive studies are necessary to define the complete cycle of *Pneumocystis* infection.

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7

***Pneumocystis* Genome Project and Genomic Organization**

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I. Introduction

It is now clear that many different species make up the genus *Pneumocystis*. To begin discussion of the genome of “*Pneumocystis*,” it is necessary to define the genome being described and to understand the similarities and differences among the members of this genus. It appears that almost every mammalian species harbors at least one species of *Pneumocystis*, which can thrive only in its specific mammalian host. These organisms are as genetically distinct from one another as other bona fide fungal species are from each other [1,2]. A more complete discussion of nomenclature and genetic diversity of *Pneumocystis* is presented in Chap. 2.

The rat, mouse, ferret, and rabbit have been the animals most commonly used to study *Pneumocystis*. Within the rat, there are at least five species of *Pneumocystis* [3]. Two of these have been characterized by karyotypic analysis and sequencing of some genes [4], while the remaining three species were identified in wild rat populations solely by amplification of a single genetic region by the polymerase chain reaction (PCR) [3]. The more characterized species occur commonly in rat colonies raised in commercial facilities and are called *P. carinii* Frenkel 1999 [5] and *P. wakefieldiae* Cushion et al. 2004 [6]. The best-

characterized of all *Pneumocystis* genomes is *P. carinii*, the focus of the *Pneumocystis* Genome Project, which is discussed below.

A. Sizes of the *Pneumocystis* Genomes

Separation of *Pneumocystis* chromosomes by pulsed-field gel electrophoresis (PGFE) techniques has provided the most accurate assessments of their genome sizes. Organisms from the rat were the first to be analyzed using variations of the PFGE technique (e.g., field inversion, CHEF, TAFE). Early separations of rat-derived organisms estimated its genome to contain about 13 chromosome-sized bands ranging in size from 0.2 to 2 Mb [7]. Improved isolation techniques and separation conditions achieved in later studies revealed the largest bands to be of rat origin and the actual chromosome numbers to range from 13 to 17, depending on the rat colony of origin [8,9]. Summation of the chromosome bands provided an estimated genome size of approximately 7 Mb for *P. carinii*. However, this is probably an underestimate of the actual size because two to three bands per profile appear to contain more than one chromosome, as evidenced by a brighter staining intensity, illustrated by the black bands in Figure 1. The genome size is more likely to be about 8.2 Mb with these additional bands included. The chromosome bands of the other species of *Pneumocystis* that inhabits rats, *P. wakefieldiae*, sum to a slightly smaller estimated genome size of 7.7 kb (Fig. 1).

Three other *Pneumocystis* genomes have been separated by PFGE techniques: mouse, human, and ferret. The human and ferret-derived organisms have estimated genome sizes of 7.0 and 11 Mb, respectively [10,11]. Better resolution of the mouse-derived *Pneumocystis* chromosomes caused us to revise our initial estimate of the size of its genome from 6.5 Mb [10] to 8.2 Mb, which is about the size of the *P. carinii* genome (Fig. 1). Alternate PFGE techniques may also warrant revisions in the sizes of the human and ferret *Pneumocystis* genomes, since there are some areas of poorer resolution within each of these profiles.

B. Electrophoretic Profiles

A single karyotypic profile (banding pattern) has been identified from the organism populations found in all hosts except the rat. The lack of karyotypic variation may be due in part to the low number of separations performed on human and ferret-derived *Pneumocystis*, which suffer from limited availability of sufficient numbers of organisms required for electrophoretic karyotyping. The most common source of human *Pneumocystis*, bronchoalveolar lavage fluid (BALF), often lacks the approximately 5×10^7 organisms necessary for a profile to be visualized by Sybr-GoldTM staining. PFGE analysis of mouse *Pneumocystis* from several mouse colonies performed in our laboratories revealed a single band pattern (Fig. 1), suggesting a situation more like that of *P. wakefieldiae* than *P. carinii* (discussed below).

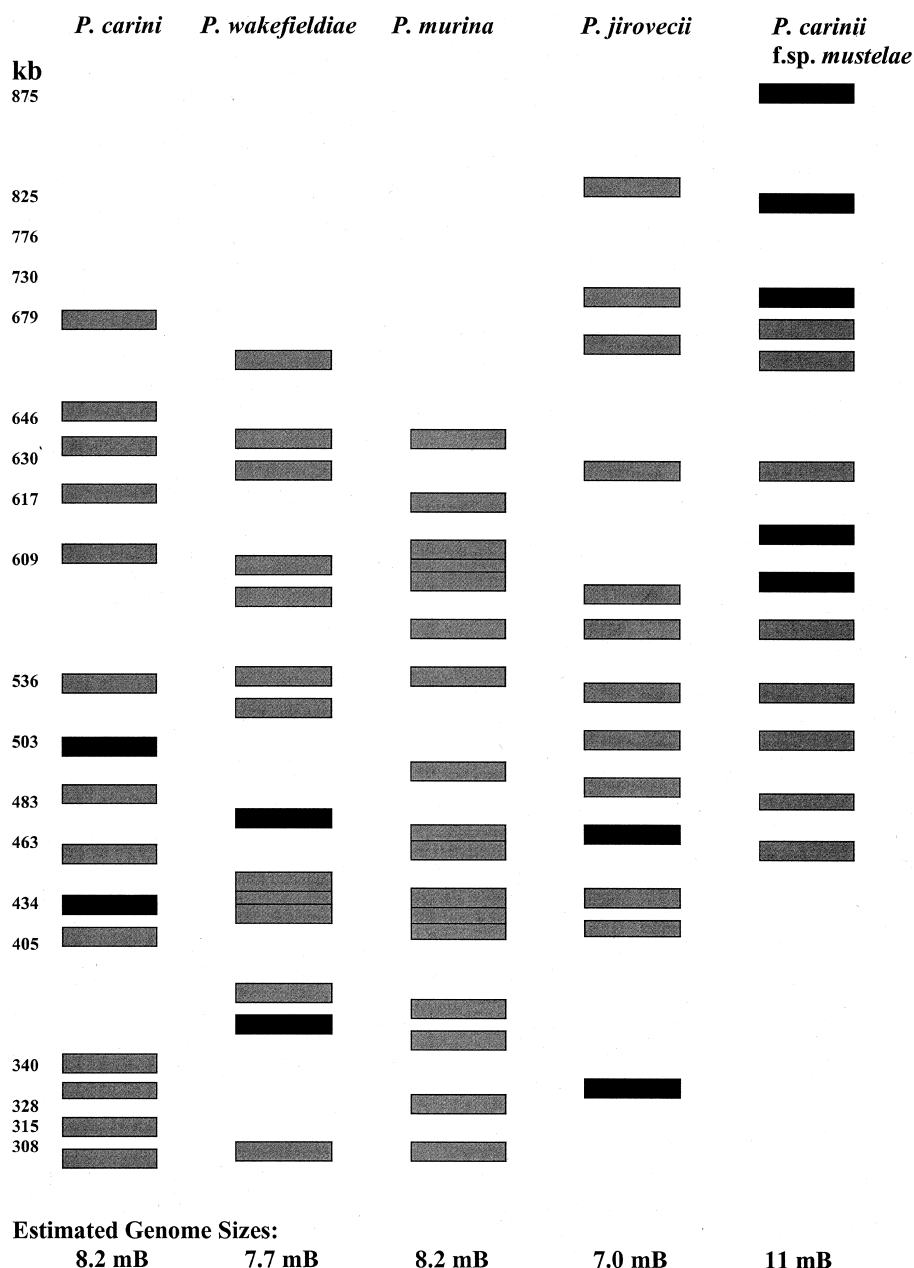


Figure 1 Schematic of electrophoretic karyotypes from *Pneumocystis* obtained from rat, mouse, human, and ferrets. Profiles were based on the migration of chromosome-sized bands using the pulsed-field technique of CHEF. Sizes of chromosomes were calculated by linear regression using concatenated lambda phage DNA(8).

P. carinii populations are the most variable in their karyotype profiles. Surveys conducted by our laboratory identified four different karyotypic patterns from 67 individual rat lung isolates obtained from 10 different colonies, using the CHEF technique [8]. Criteria for identification of a new karyotype were simply that the band pattern differed from existing patterns by the presence of one or more bands, and that this pattern was found in the lungs of at least three rats. Continued sampling of additional colonies raised the number of distinct patterns to eight [12]. Recent sampling of Taconic Farms rat colonies identified five additional unique patterns, bringing the number to 13 different profiles [13].

II. Origin of the *Pneumocystis* Genome Project

The *Pneumocystis* Genome Project was conceptualized during the 5th International Workshop on Opportunistic Protists in Lille, France, in 1997 [14,15]. As a community, *Pneumocystis* investigators decided to use the most common rat *Pneumocystis* population, *Pneumocystis carinii* f.sp. *carinii* karyotype form 1 (now *Pneumocystis carinii* Frenkel 1999) as the primary focus of the project. The genome was to be sequenced with the aid of a physical map constructed from cosmid libraries. A partial expressed sequence tag (EST) database derived from mRNA of organisms harvested from fulminant infection was to be created in the first 2 years. The community would have access to sequences and reagents generated by the project. In April 1999, the *Pneumocystis* Genome Project was funded by the National Institutes of Health for a length of 5 years. A consortium of international collaborators volunteered to participate in the project. The funded participants included Dr. Melanie Cushion as principal investigator and Drs. George Smulian and James Stringer from the University of Cincinnati as coinvestigators. The following were subcontractors: Dr. Jonathan Arnold from the University of Georgia, Dr. Chao-Hung Lee from Indiana University, and Dr. Chuck Staben from the University of Kentucky. In addition, Drs. Yoshi Nakamura, Miki Wada, and Hiroshi Mori from Tokyo University; Dr. C. Ben Beard from the CDC, Atlanta, Georgia; and Dr. Peter Philippsen, of Basel, Switzerland, were to contribute to the genome sequencing effort on an ad hoc basis. A supplemental grant from the AIDS-FIRCA Program was awarded to Dr. Ann Wakefield and Dr. Cushion in September 2000, with the goal to clone and sequence the chromosome ends of *Pneumocystis carinii* form 1.

III. EST Database

A cDNA library was constructed in Uni-ZAP XR (Stratagene Inc.) of *Pneumocystis carinii* karyotype form 1 organisms from a fulminant infection in a Long Evans rat by Dr. Smulian and used to generate most of the sequences in the EST database. The sequences were assembled using two settings of PHRED/PHRAP and one of CAP3 (<http://www.phrap.org>, <http://www.genome.cs.mtu.edu/>

sas.html). Visual examination of the sequence bins produced by each program run showed that CAP3 provided the best fit of the sequences, with fewer anomalies, and was used in subsequent analyses. The sequences were assembled into 790 contigs (assembled sequences containing more than two members) with 999 singletons (single sequences). BLASTX [16,17] analysis of the contigs and singletons revealed 578 potential homologues (e values ≤ -10), 190 low hits ($e > -10$), and 22 “no hits” to the contigs and 519 potential homologues, 223 low hits, and 255 “no hits” to the singletons (Fig. 2). A conservative estimate of the number of potential *Pneumocystis* genes in the EST database was calculated to be 1540 by simple addition of these output data.

Most of the potential homologues ($e \leq -10$) were fungal in origin (79%), with an estimated 11% contamination by rat sequences (Fig. 3). The genes of two fungal organisms made up the majority (66%) of those fungal sequences to which the *Pneumocystis* sequences had the highest homology: the fission yeast *Schizosaccharomyces pombe* (39%), and the filamentous ascomycete and model organism *Neurospora crassa* (27%) (Fig. 4). Prior to completion of the *N. crassa* genome by the Whitehead Institute (www-genome.wi.mit.edu), BLASTX analysis of the contigs showed a 67% homology to *S. pombe*, underscoring the fact that many of these matches may be database-driven and that care must be taken in reaching conclusions when a changing data set is being used. The percentage of sequences matching to *P. carinii* genes in the NCBI nr data was 17%, reflecting an increase from previous analyses due to accession of additional genes. Although the entire genome of *S. cerevisiae* has been completed, only 7% of the *P. carinii* sequences had their highest match with a gene in the yeast genome.

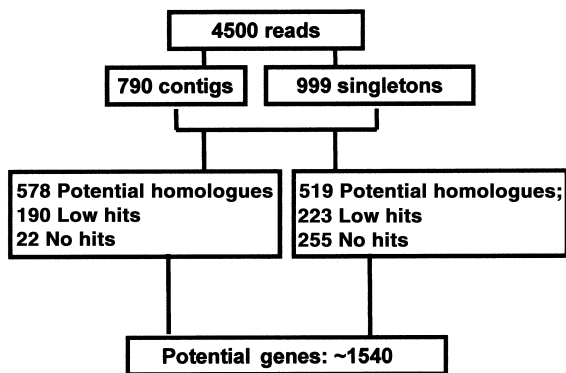


Figure 2 Schematic of computer-based analysis of expressed sequence tags from *P. carinii*. Approximately 4500 sequences from the 3' end of *P. carinii* clones from a cDNA library made from the lungs of a rat with fulminant pneumonia were analyzed using Phred, Phrap, or CAP3. These sequences were estimated to represent about 1540 genes in the *P. carinii* genome.

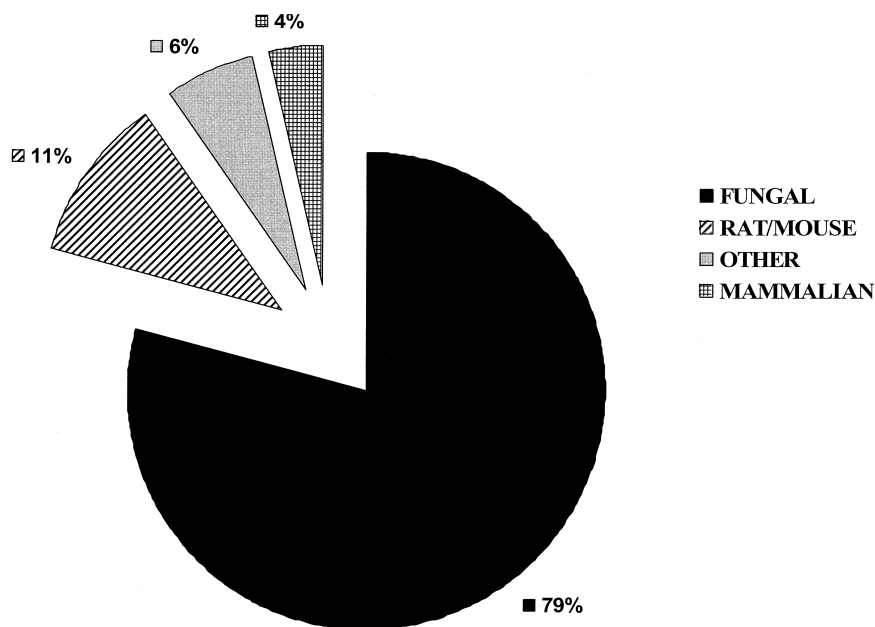


Figure 3 Breakdown of EST contig BLASTX hits by group. Consensus sequences of the 578 contigs assembled with Phrap or CAP3 were evaluated by BLASTX [16] to determine their source and affinities to genes in the NCBI GenPept database. Contig sequences were parsed into fungal, rat/mouse, mammalian, or “other” categories if their best BLASTX score was $e \leq -10$.

A multitude of metabolic cycles and cellular functions were represented by the EST contigs alone, including electron transport, glycolysis, signal transduction, mating, transcription, translation, replication, heme biosynthesis, and sterol biosynthesis. A breakdown of the metabolic function groups represented by the contig homologues using the MIPS categories (<http://www.mips.biochem.mpg.de/proj/yeast/catalogues/funcat/>) are presented in Figure 5. Comparison of the percentage of genes in the functional categories represented in the *P. carinii* genome to those in *S. cerevisiae* reveal a greater capacity for metabolic processes by *S. cerevisiae*, but, significantly, *P. carinii* appears to have an extensive array of biochemical pathways. The large number of biosynthetic cycles represented to date in the *P. carinii* genome is even more impressive when one considers that the ESTs represent only a portion (less than half) of the predicted number of genes in the entire genome, which was estimated to be about 4000 [18].

The *P. carinii* BLASTX analysis of the singleton sequences was similar but not identical to that of the contigs. Notably, parsing of the data into general groups showed a higher percentage of rat sequences in the singletons than in the

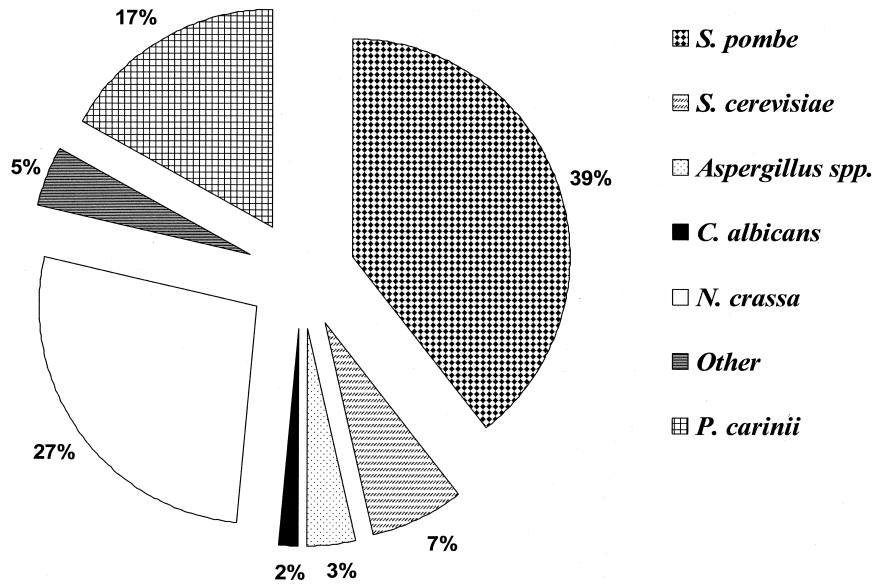


Figure 4 Breakdown of EST contigs BLASTX hits matching to fungal gene homologues. Fungal species affinities of those EST contigs with expected (e) scores of $e \leq -10$ that matched to fungal gene homologues.

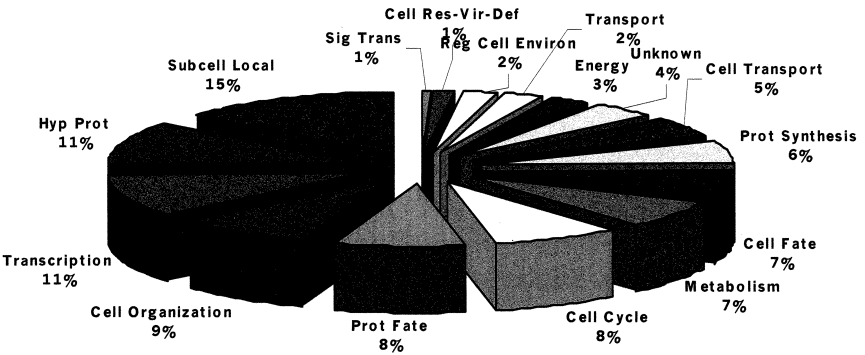


Figure 5 Functional categories represented by *P. carinii* contig homologues. The 578 contigs that matched to homologues in the NCBI GenPept data base were classified for function using the MIPS system of categorization.

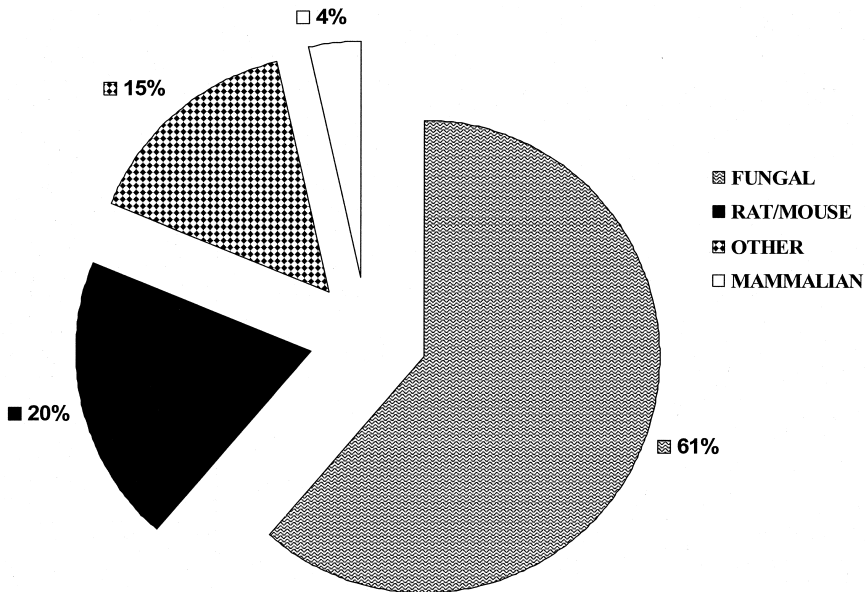


Figure 6 Breakdown of singleton BLASTX hits by group. Those sequences that did not assemble into contigs were evaluated by BLASTX (16) to determine their source and affinities to genes in the NCBI GenPept database. Singleton sequences (519 of 999) were parsed into fungal, rat/mouse, mammalian, or “other” categories if their best BLASTX score was $e \leq -10$.

contigs (20 vs. 11%) (Fig. 6). Although the majority of the potential homologues matched fungal genes in the database, the percentage was lower in comparison to the contig matches (61 vs. 79%) (Fig. 6). The difference in the number of rat sequences between the singleton and contig “hits” was not surprising, since the method of purification of the *P. carinii* organisms from the lungs favored the organism and dramatically reduced the lung cell components. This reduction in rat cells likely resulted in far less representation of highly expressed messages and thus fewer contigs. In contrast, the numbers of *P. carinii* singleton sequences with their highest hits to *S. pombe* and *N. crassa* were almost identical; 39 vs. 27% for the contigs and 39 vs. 29% for the singletons, respectively (Fig. 7). The percentage of matches to known *P. carinii* gene sequences was slightly lower in the singletons than in the contigs (12 vs. 17%), while hits to *S. cerevisiae* sequences were higher in the singleton sequences (11%) than in those of the contigs (7%). The website interface with the capacity to search the *P. carinii* ESTs can be found at <http://pneumocystis.cchmc.org>

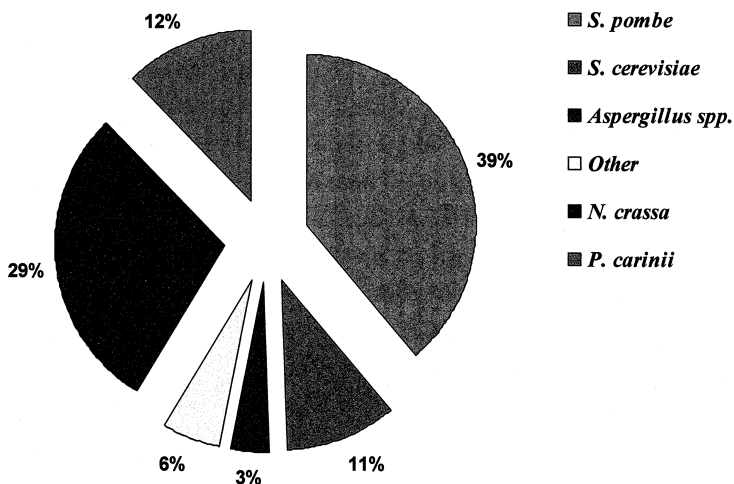


Figure 7 Breakdown of singletons matching fungal gene homologues with BLASTX. Fungal species affinities of those singleton sequences with expected scores of $e \leq -10$ that matched to fungal gene homologues.

IV. Physical Map

The initial strategy to sequence the genome of *P. carinii* was to first create a physical map of the genome using an 8 to 10x cosmid library, constructed in the vector pWEB [15], and some selected clones from a pLoristXh library. The result would provide a minimum tile as the source of DNA for shotgun libraries. The physical map was to be created by hybridization of either chromosome-specific DNA to the Southern blotted cosmid library or by hybridization of cosmid clones back to the blotted cosmid library in a "sampling without replacement" strategy [15]. This would result in bins of cosmids localized to specific chromosomes, which could then be shotgun-sequenced or arrayed into the more desirable minimum tile of approximately 220 cosmids, representing the entire genome, which could subsequently be shotgun-sequenced.

A minimum tile or physical map was only partially completed due to problems encountered with the strategy of wet hybridization (Southern blotting). The *P. carinii* genome was found to contain multiple repetitive elements that resulted in ambiguous binning of many cosmids to multiple chromosomes and prevented appropriate linkage of cosmids containing any such elements. Hybridization of the arrayed cosmid library (~2400 clones) with whole chromosomal DNA made radioactive with ^{32}P resulted in (1) signals that were overrepresented for repetitive genes such as the MSG-, MSR-, and PRoT families; and (12) signals for presumably single-copy genes that were barely above background. Increased exposure to bring out these signals resulted in overexposure of the repetitive genes and

obscuration of individual colony signals. Surprisingly, this also happened (but to a somewhat lesser degree) when randomly selected cosmid clones were used as probes and hybridized back to the cosmid library.

A “mapping by sequencing” strategy [19] was then adopted as the second strategy. Cosmid end sequences and cDNA small insert library ends were used to generate the “sequence-tagged connectors” (STCs). An additional 150 pWEB clones (seed clones) were sequenced by the shotgun method. The resulting cosmid sequences were assembled *in silico* with all the STCs, and a cosmid with the least overlap with the sequenced seed cosmid was selected next for sequencing. Sequence extension was achieved via walks initiated from the seed clones. This was an iterative process whereby each new assembly was used to choose the next set of pWEB clones to be sequenced. The data from the STCs and the wet hybridizations were imported into a novel mapping tool, ODS3, developed at the University of Georgia by Dr. Arnold and his colleagues [20]. The ODS3 is a comprehensive software package that creates an integrated genomic map at multiple levels with different visual representations. The major features are: (1) integrated mapping and sequencing output, (2) new algorithms for map building and map integration, (3) hierarchical views of the data, (4) web browser access of data, (5) object-oriented design, (6) hybridization matrix view of the physical map, and (7) support for GIF file graphics export.

A physical map based on 5280 cDNA clones, ~2500 cosmid clones, 3720 cosmid end sequences, and shotgun sequences from 150 seed cosmids was constructed by ODS3. It was estimated that ~55% of the genome was covered [20]. Dr. Arnold and colleagues created a searchable web interface for access of the physical map information and initial BLAST analysis of the *P. carinii* genomic sequence reads, generated by the University of Georgia, at <http://gene.genetics.uga.edu/>.

Limitations to the construction were the precise placement of the repetitive gene families and lack of differential filtering capacity coupled with choice of filter sets. In some cases, sequences that may actually be from the *P. carinii* genome but matched within the threshold ($e < -5$) to mammalian genes could be excluded. These problems are not unique to ODS3 and the solution awaits further software improvements.

V. Genomic Organization

The pWEB cosmid 15A6 was sequenced, gaps closed by PCR, and assembled by Dr. George Smulian using CAP3 [18]. The cosmid DNA sequence was localized to chromosome 1 (the largest) of the form 1 chromosome profile at about 700 kb in size. Fifteen genes were identified in the 32,083-bp insert; 6 on the Watson strand and 9 on the Crick strand. Genes contained 1 to 7 introns, averaging 31 to 235 bases in length. The gene density was calculated to be 1 gene per 2139 bases for an estimated 3740 genes in the genome, based on an 8-Mbp genome

size. Until recently, this represented the longest continuous genomic sequence from *P. carinii*. However, progress in the assembly of the *P. carinii* genome has produced contigs greater than twice the size of cosmid 15A6, and it is anticipated that additional data on gene density and intron usage will soon be available as result of the application of gene-finding tools and assembly of the genomic sequences with the annotated EST sequences. The reader is advised to check the website frequently for updates: <http://pneumocystis.cchmc.org>

To better understand the structure of the telomeric ends of the *P. carinii* chromosomes, clones from the pWEB library were selected by hybridization of the library with telomeric probes and other repetitive gene family sequences. To date, six pWEB cosmids have been completely sequenced by the Sanger Institute: <http://www.sanger.ac.uk>. These cosmids—18A9, 21H1, 17D7, 3G5, 11H12 and 1B2, and 22C8—contained members of the *msg*-, *prt*-, and *msr*- gene families and subtelomeric and telomeric sequences. The array structures in each cosmid were distinct from one another and are described in detail in Refs. 21 and 22. The data are summarized here.

Each cosmid began with telomeric repeats (TTAGGG), followed by subtelomeric regions that ranged between 2 and 8 kb in length, followed by an array of genes 13 to 18 kb in size containing variable numbers of members of three repetitive gene families—PRT1, MSR, and MSG—and ending in unique DNA sequence that permitted mapping of the arrays to individual chromosome bands on CHEF gels. Most of the arrays (5 of 6) contained six genes of the PRT1, MSR, and MSG families arranged in head-to-tail orientation with 3' ends pointed toward the telomere. A single copy each of MSR and an MSG gene were present in the other clone. The PRT1-MSR-MSG was not the only gene-repeat structure present in these cosmids. One cosmid (17D7) had an MSG-MSG and another (3G5) had three MSG genes in a row. A total of 13 MSG genes, 11 MSR, genes and 8 PRT1 genes were present in these arrays, with an average number of MSG genes per array of approximately two.

Mapping of the cosmids using unique DNA sequences as radioactive probes to Southern blots of *P. carinii* chromosomes separated by CHEF resulted in the following representation in the genome: cosmid 18A9, contained a sequence that encodes a protein related to the product of the *Aurl* gene of other fungi (AAB06940), localized to a single PFG band 400 kb in size; cosmid 21H1 starts with a sequence that encodes a protein weakly related to a rhoptyr protein from malaria species (AAB41263) and mapped to a single PFG band of ~690 bp in size; cosmid 17D7 begins in an ORF that encodes a P-type cation-pumping ATPase (NP_595246), and mapped to a single PFG band 600 kb in size; cosmid 3G5 starts with two presumptive genes, *Nmp* and *Map*, both of which hybridized to a single PFG band, which was approximately 250 kb in size; cosmid 1B2 did not begin in unique DNA, but restriction mapping identified 13 other cosmids related to 1B2 in the pWEB library, and one of these cosmids (11H12) contained an insert that began with a sequence (*Chi*) that encodes a peptide weakly related

to fungal proteins needed for chitin synthesis (NP_013434). Probes made from the *Chi* sequence mapped to a single PFG band 400 kb in size; cosmid 22C8 began in unique DNA that encodes a protein very similar to the U2 snRNP spliceosome subunit protein of other fungi (NCBI Conserved Domain Database CD: COG5181.1, HSH155) and mapped to chromosome 4 (550 kb). The pWEB clones hybridized to one end of 4 of the 16 chromosomes in the *P. carinii* genome, whereas 2 clones were mapped to the 400-kb band, suggesting that both ends of this chromosome have been identified.

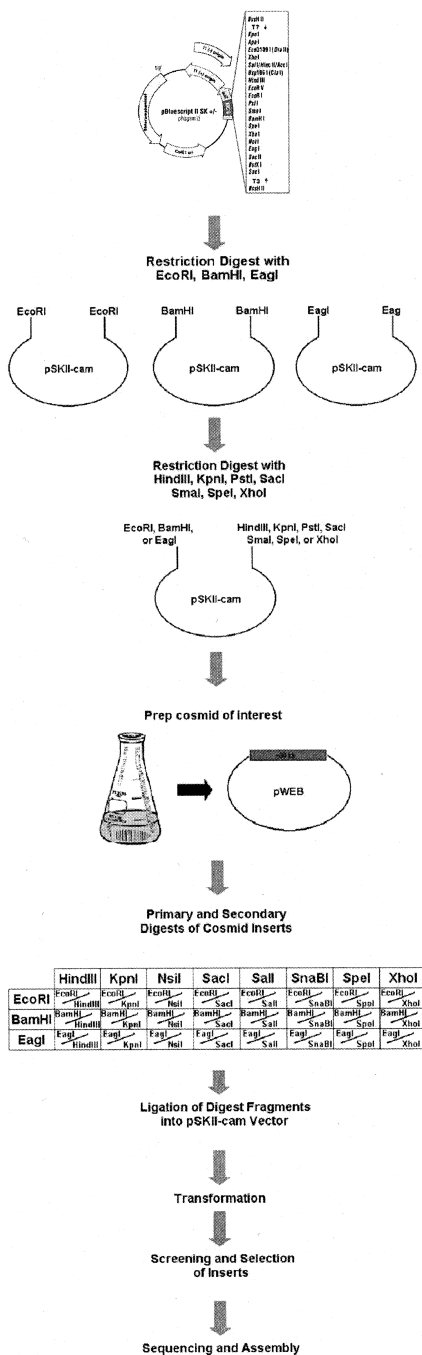
VI. Sequencing of the Genome

At the time of this writing, over 9 million base pairs of high-quality sequence had been produced by the *Pneumocystis* Genome Project. About 50% have been assembled into contiguous sequences of 1.4 Mb, representing a 3.3X coverage of the genome. The unassembled sequences represented approximately a 0.8X coverage of the remaining genome. Together, these values represent approximately 65% of the genome that has been sequenced, without considering the component of sequence supplied by the EST database. The majority of this sequence has been generated by blunt-end shotgun cloning of cosmid DNA and “sticky-end” cloning of the same. More recently, a major advance in sequencing of the *P. carinii* genome has been made using an approach that constructs shotgun libraries by nanocloning [23] of chromosomal DNA excised from low-melt CHEF gels. These approaches are discussed below.

Using the “walking” type of strategy to provide genomic sequence from shotgun libraries of selected cosmid clones, as described in Section IV, two major problems were encountered: (1) the vector (pWEB) was preferentially cloned into the blunt-end vector pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA); and (2) portions of the *P. carinii* genome represented in the pWEB library clones appeared to be preferentially cloned, resulting in a high density of sequence in some regions and absence of sequence in others.

A new strategy for sequencing was devised that addressed these issues. Figure 8 shows a schematic of the process. The pBluescript SK II vector (Stra-

Figure 8 Schematic of sticky-end cloning of the *P. carinii* genome, pBluescript SKII was modified by insertion of a chloramphenicol resistance gene (CAM). To produce clones of *P. carinii* in the cosmid vector, pWEB, the CAM vector was first digested with EcoRI, BamHI, and EagI; then each vector was digested with HindIII, KpnI, PstI, SacI, SmaI, SpeI and XhoI. pWEB cosmids of interest were grown in Luria broth with 50 µg/mL carbenicillin and isolated from bacteria using the Qiagen Large Insert Preparation Kit. The cosmids were then digested with the same restriction enzymes as outlined for the CAM vector. The digested fragments were ligated into the CAM vector and transformed into *E. coli* cells made competent, plated, and selected on LB agar containing 35 µg/mL chloramphenicol.



tagene, La Jolla, CA) was modified to contain the gene for chloramphenicol resistance (pSKII-cam) as a means to inhibit growth of plasmids containing portions of pWEB vector carrying resistance genes for ampicillin or kanamycin. The vector and the cosmids were digested with a series of restriction endonucleases to create compatible sticky ends to overcome the cloning bias. After ligation and transfection into *Escherichia coli*, the plasmids were plated onto selective media and subsequently purified and digested to screen for inserts of novel size and for the presence of the vector by Southern blotting and probing with pWEB. After sequencing and assembly with ARACHNE, it was found that 70% of the sequences produced by the new cloning strategy were included in the assembly, in contrast to 53% of the blunt-end vector sequences. The remaining sequences were excluded from the assembly due to poor base quality or contamination with vector. This new strategy was more efficient, allowing for a greater amount of useful sequence to be generated, but it was also more time-consuming and expensive.

The most recent approach to sequencing of the *P. carinii* genome has been to prepare shotgun libraries from chromosomal DNA separated in low-melt agarose CHEF gels. This strategy had been attempted early on in the project, but libraries were of poor quality due to problems of insufficient DNA and perhaps other inhibitors that interfered with efficient cloning. The chromosome libraries were prepared by Lucigen Corporation (Middleton, WI) using DNA that was not exposed to ultraviolet wavelengths and a blunt-end cloning vector, pSMART LCKAN. This vector is a low-copy plasmid that lacks a promoter or an indicator gene, so transcription across the insert is eliminated, thereby reducing cloning bias. After hydrodynamic shearing, the *P. carinii* DNA was end-repaired and special linkers were added to facilitate amplification with the high-fidelity polymerase Vent for 25 cycles. The DNA was then size-fractionated and ligated with subsequent electroporation into competent *E. coli* cells. Results from the first 192 sequence reads of chromosomes 13 to 15 (prepared as one library) are shown in Figure 9. Summation of the lambda, rat, and bacterial percentages provides an 8% overall contamination rate. The lambda sequences arose from contamination of the processing apparatus, while the rat and bacterial sequences came from the rat lungs. Although PFGE enriches for the chromosomal *P. carinii* DNA, DNA from other sources, especially rat lung cells, migrates throughout the entire gel with localization toward the top of the gel above the separated chromosomal bands (compression zone) or toward the bottom of the gel as degraded DNA. The integrity of the library can be seen in the percentage of sequences matching those of *P. carinii* genes in the NCBI nr database (23%) and the 17% of sequences with homology to genes from other fungi. Within those sequences matching with known *P. carinii* genes, MSG and related genes were most prevalent but not overrepresented, and genes that have previously been localized to these chromosomes by Southern hybridizations were present—for example, eukaryotic release factor 3. Most of the sequences (52%) hit to other organisms, such as *Arabidopsis*

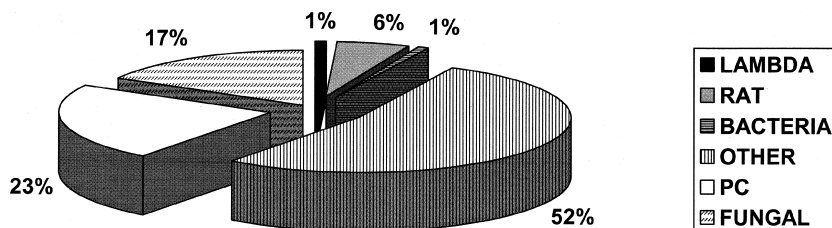


Figure 9 Analysis of Lucigen, Inc., library of chromosomes 13 to 15. Sequences generated from the library constructed by Lucigen, Inc., of chromosomes 13 to 15 were analyzed by BLASTX. Sequences were parsed into fungal, rat/mouse, mammalian, or “other” categories if their best BLASTX score was $e \leq -10$.

or other plants, suggesting the *P. carinii* may have genes distinct from other fungi.

VII. Mitochondrial Genome

The mitochondrion is a major target for secondary therapy of *Pneumocystis* pneumonia. Atovaquone targets the bc_1 complex of the electron transport chain [24,25], and one of the mechanisms of action of pentamidine has been suggested to be mitochondrial inhibition [26]. The mitochondrial genome of *P. carinii* has been estimated to be ~40 to 50 kb, based on migration through pulsed-field gels [10]. Although sequencing of the mitochondrial genome was not a goal of the *Pneumocystis* Genome Project, some sequence has become available, as is the case in many genome projects. Sequencing of the ends of the pWEB identified sequence homologous with fungal mitochondrial genes. One cosmid, 12c7, (~12,000 bp) was sequenced and partially assembled using CAP3. Three contigs of 3194-, 8146-, and 988 bp were constructed and analyzed by WU BLAST to nucleotide and protein databases. Genes identified in the *P. carinii* mitochondrial genome included cytochrome B, NADH dehydrogenase subunits 4 and 5, cytochrome oxidase 3, large subunit rRNA, and ATPase subunit 6 and 9. These genes and those previously identified in GenBank suggest that the mitochondrial genome of *P. carinii* resembles most fungal mitochondrial genomes except that of *S. pombe*, which is much smaller, at 19 kb, and lacks the NADH genes. The migration of the mitochondrial genome on CHEF gels may be suggestive of a linear rather than a circular configuration.

VIII. Available Reagents and Informatics

The UniZap cDNA library constructed for the *Pneumocystis* Genome Project and used to generate the EST database is available from the American Type Culture

Collection (www.ATCC.org). The pWEB cosmid library has not yet been deposited, but clones are available upon request from Melanie. Cushion@Uc.Edu. The website at which the genetic data are available is <http://pneumocystis.cchmc.org>. New assemblies are uploaded on an approximate weekly basis. The *Pneumocystis* genome is being annotated by Dr. Jarek Meller, Children's Hospital Research Center, Cincinnati, OH, and these data will become available on the *Pneumocystis* Genome Project website and on Dr. Meller's own website with supplemental information: <http://folding.chmcc.org>

IX. Naming Conventions

As additional gene sequence becomes available from both the *Pneumocystis* Genome Project and through the sequencing efforts of individual laboratories, a standardized naming convention will greatly facilitate annotation and curation efforts. Putative genes identified by the genome sequencing effort will be assigned interim identifiers based on their genomic location and a schema similar to that used by the *S. cerevisiae* genome sequencing effort. The identifier will follow the convention PCA001, PCA002, PCA003 .|. ., where PC identifies the organism origin as *Pneumocystis carinii*, A indicates its location on chromosome 1, and 001, 002, 003 .|. ., as adjacent genes within a contiguous sequence (chromosome, contig or cosmid). In addition to the identifier, individual genes may be assigned gene names using the convention proposed by Stringer and Cushion [10]. Association of the gene identifier (e.g., PCA001), the gene name (e.g., *gsh1*), and the ESTs within the database (e.g., s35f9) will be provided at the *Pneumocystis* website: <http://pneumocystis.cchmc.org>. As proposed, genes should be assigned a three-letter lowercase italic name (*xyz*); the putative gene product should be designated by a three-letter roman name with the initial letter capitalized (*Xyz*). Genes should be assigned the name of the *S. cerevisiae* orthologue (where gene function is often best characterized), with the understanding that the function of the gene product in *P. carinii* may ultimately be shown to be distinct from that of its homologue. Gene families will be identified by a three-letter roman name capitalized (e.g., MSG family), with individual genes designated *msg1*, *msg2*, *msg3* .|. . and the predicted proteins *Msg1*, *Msg2*, *Msg3* .|. . Adherence to a standardized naming convention will greatly assist later efforts to correlate all available data and to assemble data within databases.

X. Conclusions

The historic inability to propagate *P. carinii* outside the mammalian lung has hindered progress in most research areas. When the *Pneumocystis* Genome Project was initiated, only a few gene sequences had been published, and it was felt that

the only way to make progress in any scientific area was to sequence the genome as a means to begin to understand its basic properties.

The *Pneumocystis* Genome Project began as an international initiative, and the genetic data it has produced represent a significant resource to the same community as well as to scientists from other disciplines. A number of studies using the project data have already been published. Genes that encode pivotal steps in the sterol biosynthetic pathways have been cloned, expressed, and biochemically characterized [27,28]; macroarrays of gene sequences were used to probe the effects of drugs on *P. carinii* metabolism [29], and new analytical tools were developed for physical mapping and workflow [20,30,31].

The significance of the project is only starting to be realized. As the assemblies lengthen and more genes are identified, investigators in the *Pneumocystis* community and the scientific community at large will have access to gene sequences of interest. Not only will we gain an appreciation of the metabolic capacity of this organism family, but with gene sequence in hand, direct progression to functional analysis will proceed at a much faster rate. Completion of the Genome Project (estimated to be early 2005) will pave the way for creation of microarrays and other functional assays (e.g., heterologous expression systems), which will further push forward research on these once intractable organisms.

The mouse and human *Pneumocystis* genomes will be sequenced by investigators at the Whitehead Institute, MIT, under funding provided by the Fungal Genome Initiative (http://www-genome.wi.mit.edu/seq/fgi/FGI_whitepaper_Feb8.pdf). Genomic and genetic comparisons of the rat, mouse, and human *Pneumocystis* should provide insights into the host-specific factors required by each species and genetic characteristics responsible for the observed differences in their biological functions.

List of Abbreviations

- ARACHNE: a software program that assembles DNA sequences into larger tracts of sequence
- ATCC: American type culture collection
- BALF: bronchoalveolar fluid
- BLASTX/N: basic local alignment search tool, a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA
- CAP3: a software program that assembles DNA sequences into larger tracts of sequence
- cDNA: complementary DNA
- CHEF: contour-clamped homogeneous electrical field
- Contigs: an assembly of two or more DNA sequences
- EST: expressed sequence tag

Lorist 6Xh: a cosmid vector

Mb: megabase pairs

MIPS: Munich Information Center for Protein Sequences

MSG: the major surface glycoprotein gene family in *Pneumocystis carinii*

MSR: the MSG-related gene family in *Pneumocystis carinii*

NCBI: National Center for Biotechnology Information

Nr: nonredundant

pBluescript SK II vector: small insert cloning vector (Stratagene La Jolla, CA)

PCR: polymerase chain reaction

pCR4Blunt-TOPO: a cloning vector that accepts blunt ends and PCR products (Invitrogen, Carlsbad, CA)

PFG: pulsed-field gel

PFGE: pulsed-field gel electrophoresis

PHRAP: a software program that assembles DNA sequences into larger tracts of sequence

PHRED: a base editing program that assigns quality scores to nucleotide sequence

PRT: subtilisin protease gene family in *Pneumocystis carinii*

pSMART LCKAN: a cloning vector that accepts blunt end inserts and replicates in low copy number (Lucigen Inc., Madison, WI)

pWEB: a cosmid vector capable of maintaining large inserts of foreign DNA (up to 40 kb)(Epicentre Technologies, Madison, WI)

Singletons: unassembled DNA sequences that exist as the solitary sequence read

TAFE: transverse alternating-field electrophoresis

Uni-ZAP cDNA library: a library constructed from DNA made complementary to mRNA (Stratagene, La Jolla, CA)

WU Blast: Washington University Basic Alignment Search Tool

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8

The Neonatal Period

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I. Introduction

A. History

Pneumocystis was first described by Chagas in 1909 in guinea pigs, small primate species, and a 4-month-old malnourished infant who had died in the area of Brazil where Chagas first discovered a trypanosome that was infecting railroad workers [1,2]. In the early 1950s, it was found by Vanek and Jirovec that the interstitial plasma cell pneumonia that had been reported in sporadic outbreaks in Europe for nearly 20 years was due to *Pneumocystis* [3–5]. These outbreaks involved mostly infants who had a predisposing condition, such as malnourishment. Endemic interstitial plasma cell pneumonia was the primary cause of death in European orphanages between 1950 and 1960, with a mortality rate between 10 and 60% [5,6]. This was thought to be a “European” disease until 1955, when cases showed up in children in the United States [7,8]. In 1964, an outbreak of *Pneumocystis* pneumonia was reported in an orphanage in Shiraz, Iran [9]. Sixty two children suffering from marasmus died from interstitial plasma cell pneumonia caused by pneumocystosis over a 7-year period, most being under the age of 1 year [6]. With the start of the AIDS epidemic in the early 1980s, the incidence of PcP in children once again became a problem. Significant efforts have been

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made to understand the underlying immunodeficiency diseases that predispose young children to PcP. However, more recently, there have been reports that *Pneumocystis* has been found in the nasopharyngeal aspirates of immunocompetent children suggesting that children may be a natural reservoir for these organisms [10,11]. Animal models of PcP have become important in determining the immunological basis for the success of the organisms in infecting infants and are discussed in detail in this chapter.

B. Predisposing Conditions

Underlying immunodeficiencies are the major predisposing factors for PcP in infants, just as they are in adults. Children diagnosed with primary immunodeficiency syndromes—such as severe combined immunodeficiency (SCID), X-linked agammaglobulinemia, or X-linked hyper-IgM syndrome—generally acquire PcP early in life [12–16]. The immunological basis for susceptibility to PcP in these children is varied and has provided important clues to the basis of host defense against *Pneumocystis*. As with adults, infants with acquired immunodeficiencies due to HIV, corticosteroid treatment, chemotherapy, and marrow and solid organ transplantation are also susceptible to PcP [17–25]. Premature infants and those born to malnourished mothers were found to be susceptible to PcP, particularly in orphanage epidemics in Europe during World War II and in war-torn Vietnam and Iran in the 1950s and 1960s [2,26]. Interestingly, children suffering from malnourishment in the absence of other immunosuppressive diseases tend to develop PcP at a very early age (less than 1 year), whereas there is little information regarding susceptibility of malnourished adults to PcP [5,6,27]. While it is clear that protein-calorie, vitamin, and trace element deficiencies can lead to immune suppression, it is not known why PcP is a particular problem among malnourished infants [28]. It is easy to speculate that infants have immature immune systems anyway, and that further suppression due to malnutrition renders them even more susceptible to opportunistic infections. However, the basis of this susceptibility has not been formally demonstrated.

A number of immunodeficiency states have been reported to predispose infants to PcP; but certainly the largest number of children who acquire PcP are HIV-positive. In spite of the renewed interest in PcP in children due to the AIDS epidemic, perhaps of most interest are the reported cases of PcP where no underlying immunodeficiency was detected. These cases are discussed below.

C. PcP in Immunocompetent Children

Primary infection with *Pneumocystis* is common in children, since over 90% have detectable antibody to the organisms by 2 to 4 years of age [29]. Although the diagnosis of PcP in immunocompetent children is rare, there have been case reports of PcP in seemingly normal, full-term infants [10,30]. However, respira-

tory infection with *Pneumocystis* may take place in infants more frequently than previously appreciated. Vargas et al. recently reported that 32% of infants recruited from an outpatient clinic in Santiago, Chile, had nasopharyngeal samples positive for *Pneumocystis* DNA [11]. Importantly, these infants were at the clinic due to episodes of respiratory infection [11]. Other studies have reported PcP in infants who did not have a diagnosed immunodeficiency disease [10]. Together, these studies suggest that infection of infants with *Pneumocystis* can be subclinical or may result in respiratory problems ranging from mild to more severe. However, the disease does not appear to be as fulminant as in infants with immunodeficiency diseases, particularly AIDS. Interestingly, infant rabbits, piglets, and foals have been reported to acquire PcP prior to weaning, even though they have no underlying immunodeficiency [31–34].

Autopsy specimens from children who died of sudden infant death syndrome (SIDS) indicate that a large proportion of them (35.1% from Chile and 14.8% from Oxford) had *Pneumocystis* organisms in their lungs, as determined by histological diagnosis [35]. This was compared to 2.9% of children who had died of causes other than SIDS. It is not clear whether *Pneumocystis* infection predispose children to SIDS or whether conditions that lead to SIDS predisposes children to PcP. This warrants further study. These recent reports of *Pneumocystis* infection in seemingly normal, full-term infants along with sporadic reports of PcP in infant animals have led to renewed interest in the epidemiology of PcP in children. In addition, there is significant interest in the immunological basis for a seemingly permissive growth environment for *Pneumocystis* in infant lungs. This issue is discussed at length in the following sections.

D. PcP in Infant Animals

Pneumocystis infection has been identified in a wide variety of mammalian species, as described elsewhere in this volume. Interestingly, there is a fairly large literature regarding reports of PcP in infant animals. As with humans, PcP has been reported in immunodeficient infant animals. Arabian foals that inherit combined immunodeficiency are particularly susceptible to PcP [36]. These infections are frequently associated with other infectious agents, such as adenovirus and *Rhodococcus equi* [36,37]. More recently, there have been reports of PcP in foals that have no apparent underlying immunodeficiency [38]. These animals tended to succumb to infection rapidly after clinical symptoms were detected unless *Pneumocystis* organisms were identified and treatment was initiated [34,39].

It has been also found that infant rabbits and pigs can develop PcP spontaneously without an apparent underlying immunodeficiency state [31,33,40]. Interestingly, infant rabbits develop PcP around the time of weaning and resolve the infection 2 to 3 weeks later [31]. In contrast, endemic PcP has been reported in herds of pigs, resulting in significant deaths among infants that developed respira-

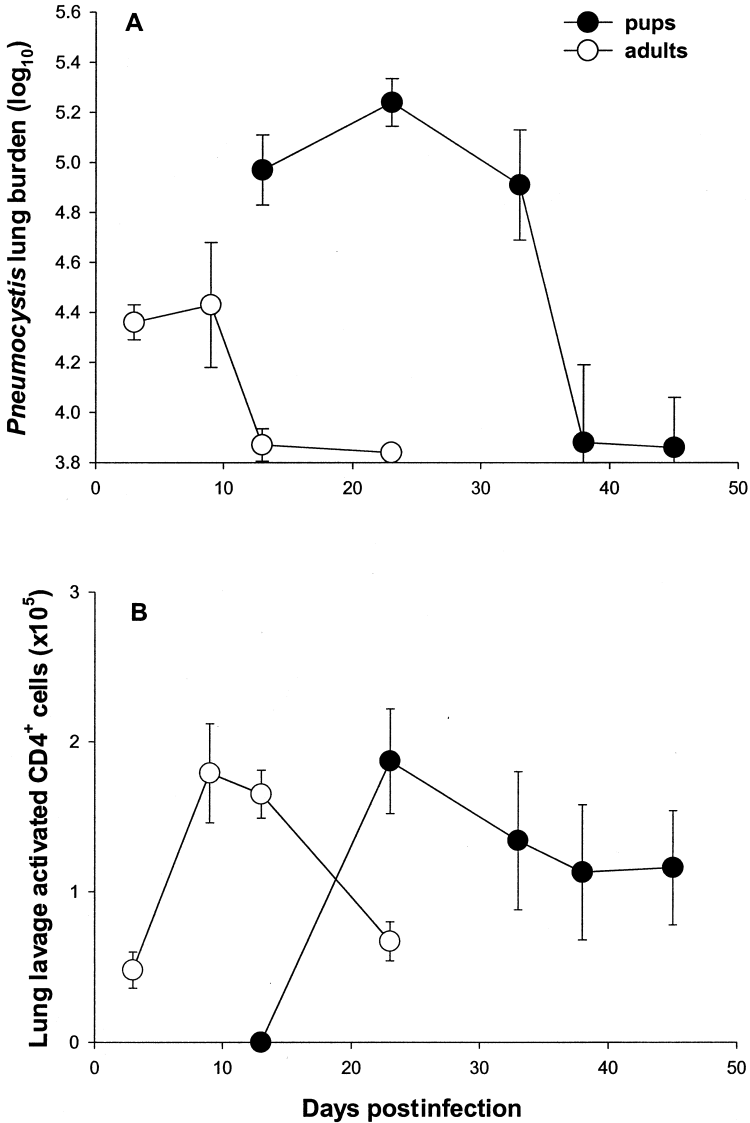


Figure 1 Delayed clearance of *Pneumocystis* infection in neonatal mice corresponds to delayed infiltration of activated ($CD4^+CD45RB^{lo}$) cells into the alveolar spaces. A. Lung *Pneumocystis* burden was determined microscopically using Diff Quik–stained cytospin preparation. B. Activated $CD4^+$ cells obtained from lung lavages were quantitated using flow cytometry. Data represents the mean $\pm$ SD of four to five mice per group. (Adapted from Ref. 41.)

tory distress as a result of their infections [32,33]. These animals were also seemingly healthy prior to acquiring PcP, although it cannot be ruled out that they could have been immunosuppressed due to stresses such as overcrowding. Together, these reports, along with those from *Pneumocystis* infection in immunocompetent human infants, suggest that newborns are significantly more susceptible to PcP than adults. The basis for this susceptibility could be an immature immune system, lack of maternal antibody, or a lung environment that is not conducive to host responses. Although useful information has been gleaned from the case reports of PcP in infant foals, piglets, and rabbits, these animals are not the best models for studying immune responses to *Pneumocystis* in neonates since they are either expensive to maintain or immunological reagents are not readily available. We have chosen to use an infant mouse model of infection to examine the basis of the immune response to *Pneumocystis* in neonates. Mice inoculated with *Pneumocystis* as neonates (24 to 72 hr of age) fail to develop an immune response in the lungs until approximately 3 weeks of age. At around weaning age for mice (3 weeks old) there is an infiltration of activated CD4⁺ T cells into the lungs followed by clearance of the infection by about 6 weeks of age (Fig. 1) [41]. In contrast, adult mice normally mount an immune response to *Pneumocystis* within several days and clear *Pneumocystis* within 3 weeks of inoculation [41]. The reason for this delayed immune response to *Pneumocystis* in infants is discussed below.

II. Transmission

Transmission of *Pneumocystis* is covered in another chapter of this volume; however, transmission in neonates has been somewhat controversial and is therefore discussed briefly here. It has been recognized for some time that most children develop antibodies to *Pneumocystis* by the age of 2 years, indicating that the organisms are encountered early in life [29]. In 1962, Pavlica first reported that *Pneumocystis* was found in the lungs of a stillborn fetus [42]. In 1991, Mortier et al. also reported maternal-fetal transmission in an infant infected with HIV [43]. Unfortunately, both of these reports relied on histochemical analysis of lung sections rather than more definitive methods of diagnosing [44]. Consistent with the notion that *Pneumocystis* can be transmitted transplacentally in humans, it has been reported that *Pneumocystis* was transmitted in utero in rabbits [45]. These data were based on finding *Pneumocystis*-specific PCR products from the lungs of 15- and 26-day-gestation fetuses as well as immunofluorescence data using lung tissue [45]. The authors suggest that the placenta becomes more permeable as the stage of pregnancy advances, so that *Pneumocystis* is able to cross the placenta at later stages [45].

Experiments using mouse and rat models have failed to detect vertical transmission of *Pneumocystis* [46–48]. Ito et al. using histochemical and immuno-

fluorescence assays, examined SCID mice taken by cesarean section from *Pneumocystis*-infected dams and were unable to demonstrate transmission of the organisms [46]. Hong et al. reported that *Pneumocystis* was found in the lungs of 1-week-old rats born to infected, immunosuppressed dams [48]. *Pneumocystis* was detected by histochemical techniques as well as in situ hybridization; however, it was not possible to eliminate the possibility that the infant rats acquired *Pneumocystis* infections postnatally, since they were housed in cages with infected dams. In fact, Icenhour et al. have demonstrated, using PCR specific for *Pneumocystis* DNA, that rats do not acquire infection in utero [47]. However, within 2 hr of birth, 80% of pups born to infected dams were positive for *Pneumocystis*-specific DNA obtained by oral swabs. These observations suggest that acquisition of *Pneumocystis* by infants is due to transmission of the organisms from the environment, in this instance by intimate contact with their infected mothers [47]. The discrepancy between the observations of vertical transmission occurring in humans and rabbits but not in rats or mice could be due to differences in the integrity of the placentas, as might be inferred from the work of Cere et al. [45]. But in utero transmission is dependent on extrapulmonary organisms existing in the mothers, a phenomenon that is rarely reported. Clearly, *Pneumocystis* infections can be acquired very early in life, but it is not clear that in utero acquisition is an important mode of transmission.

III. Neonatal Immune Function

Our understanding of the nature of host defense to *Pneumocystis* has been enhanced significantly over the past 20 years. Much of this is due to the intense scrutiny afforded *Pneumocystis* since the beginning of the AIDS epidemic. We have also gained a significant amount of knowledge about immune responses in general due to the development of new technologies and reagents. In addition, the generation of new animal models has propelled the study of immune responses to infection forward as at no other time in history. The nature of the immune response to *Pneumocystis* in adults is detailed in several chapters of this volume. In any event, it is clear that the immune response to infection is different in neonates than in adults. As mentioned previously, there is a 3-week delay in clearance of *Pneumocystis* from the lungs of mice infected as neonates compared to adults [41,49,50]. Furthermore, infant rabbits and humans seem to harbor *Pneumocystis* organisms in the lungs [11,31]. Neonates generally have an unresponsive immune system—a fact that has been attributed to “immaturity.” This could refer to the function of lymphocytes, antigen-presenting cells, and/or phagocytic cells, or it may be that the pulmonary environment in neonates is not conducive to generating immune responses. Although there is still much that is not known about how infants handle *Pneumocystis* infection, animal models have recently provided new insights into why neonates seem to take longer than adults to clear the infection. Most of what is known about host defense against *Pneumocystis*

in neonates has been learned from a mouse model of infection; therefore much of the rest of this chapter concentrates on this model.

A. Innate Immunity

Actual killing of *Pneumocystis* is likely due to phagocytosis and degradation of organisms by alveolar macrophages, as described in detail elsewhere in this volume. Although innate defenses are not sufficient for clearing *Pneumocystis* in the absence of lymphocytes, it has been demonstrated that macrophages are required for clearance [51]. There is little information regarding the function of alveolar macrophages in neonates; however, it has been reported that the ability of alveolar macrophages from newborn rats to phagocytose bacteria is as good as or better than that of adults [52]. Similarly, we have found that the ability of alveolar macrophages from infant mice to phagocytose fluorescent beads or fluorescently labeled *Pneumocystis* organisms was not different from that of adult mice [49]. Even so, *Pneumocystis* grows seemingly unchecked in the lungs of infant mice for a prolonged length of time, suggesting that activation of macrophages may not be sufficient for actual killing of the organisms. This likely requires a T-cell response, which is significantly delayed in the lungs of mice infected as neonates vs. those infected as adults [41]. Interestingly, it has been reported that alveolar macrophages from newborn rats produce similar amounts of TNF- α in response to lipopolysaccharide (LPS) as do alveolar macrophages from adult rats [52]. In addition, upregulation of mRNA expression for IL-12, IL-18, and TNF- α in response to LPS was not different in cultured alveolar macrophages from newborn and adult rats [52,53]. However, we have found that there is very little mRNA expression of TNF- α or IFN- γ in the lungs of neonatal mice infected with *Pneumocystis* [49,50].

It is possible that *Pneumocystis* is quite good at evading neonatal host defenses or, alternatively, that the neonatal lung environment may not be adequate for generating a response. It appears that *Pneumocystis* is able to grow in infant lungs without causing severe symptoms or posing a danger to the infants. Certainly, neonatal mice are capable of mounting rapid responses to more threatening infectious agents, such as *Streptococcus pneumoniae*. Within 4 hr of intranasal challenge, neonatal mice have a significant alveolar infiltration of neutrophils [64]. This suggests that perhaps a response to *Pneumocystis* is not mounted until the organisms reach a critical level that it is dangerous to ignore. This strategy would delay the response in postnatally developing lungs, where inflammation could be more dangerous to the infant than the infectious agent itself.

A component of innate immunity that may have importance for host defense is the family of surfactant proteins present in the lungs. The role of various surfactant protein molecules on host defense to *Pneumocystis* is discussed at length elsewhere in this volume. However, the underdeveloped lungs of premature

newborns are deficient in surfactant [54]. Seventy-five percent of surfactant protein A is made during the last quarter of gestation, and surfactant D is first detected in the second trimester and continues to increase postnatally [54]. It has recently been shown that surfactant protein A-deficient mice, compared to wild-type mice, have increased susceptibility to *Pneumocystis* [55]. Furthermore, it has been reported that surfactant D facilitates phagocytosis of *Pneumocystis* by alveolar macrophages [56]. It is possible that a deficiency in surfactant proteins due to pre-mature birth contributes to the susceptibility of these infants to PcP [5]. However, this has not been formally demonstrated.

B. Cellular Immunity

It has been long recognized that suppression of lymphocyte function can result in susceptibility to PcP [5,36,57]. Interestingly, the function of the cellular immune system in infants has been described as “immature”, in that T cells have been shown to be easily tolerized, that they do not produce cytokines with the same efficiency as adults, and that they proliferate poorly to stimulation through the T-cell receptor (TCR) [58]. A logical deduction from this information might be that infants would be susceptible to PcP. However, there are very few reports of immunocompetent infants with PcP, though it has been shown more recently that infants can be carriers of the organisms without developing apparent pneumonia [11]. It has been shown from mouse studies that inoculation of *Pneumocystis* into infants results in the growth of the organisms without an apparent immune response until the mice are about the age of weaning (3 weeks old). This corresponds to delayed infiltration of lymphocytes into the alveolar spaces of mice. As shown in Figure 1, the delay in clearance of *Pneumocystis* corresponds to delayed T-cell responses into the alveolar spaces. This delay in lymphocyte responses in infant mice compared to adults may be due to immaturity of T cells or inefficient antigen presentation.

Antigen Presentation

There has been a recent interest in the function of dendritic cells, an important antigen-presenting cell (APC) in neonates. Data from neonatal rats indicates that alveolar dendritic cells do not express adult-level staining patterns for MHC class II until the age of weaning [59,60]. Furthermore, dendritic cells from neonatal rats had reduced antigen-presentation activity in mixed lymphocyte reactions and were less able to migrate into the airways in response to heat-killed *Moraxella catarrhalis* than those of adult rats [59]. Consistent with this finding, we reported that, in neonatal mice infected with *Pneumocystis* there are decreased numbers of CD11c⁺ dendritic cells in the lungs compared to adults and that these cells express lower levels of MHC class II than those of adult cells [49]. In addition, although the number of dendritic cells in the draining lymph nodes was equivalent

in neonates and adults at day 8 postinfection, the expression levels of MHC class II on neonatal cells was about one-third of that of adult cells [49].

When dendritic cells move into the lungs, they are highly phagocytic but poor antigen presenters. They are dependent on inducible cytokines—such as RANTES, MIP-1 α , MIP-1 β , and MCP-1—for migration from the circulation into the site of infection [61]. Once in the lungs, the immature dendritic cells mature from being phagocytic to being APCs. At the same time, they upregulate CCR7 and downregulate CCR1, -2, and -5, which targets them to the draining lymph nodes in response to EBV-induced molecule 1 ligand chemokine (ELC/MIP-3 β), where they present antigen to T cells [62,63]. There is a delay in the expression of RANTES, MIP-1 α , MIP-1 β , and MCP-1 mRNA in the lungs of mice infected with *Pneumocystis* as neonates compared to adults, which may result in delayed migration of dendritic cells into the lungs, delayed antigen presentation to T cells, and delayed clearance to the organisms [49]. In addition, preliminary data indicate that mRNA expression of CCR7 is also depressed in infant mice infected with *Pneumocystis* compared to infected adults (Qureshi and Garvy, unpublished observations). Consistent with this finding is a report of PcP in an immunocompetent foal whose peripheral CD3⁺ T cells were normal, as were the peripheral blood antibody levels [34]. These authors speculated that the T cells may have been normal but antigen presentation or cytokine production in the lungs was not. Together, these data suggest that the function of dendritic cells from neonatal mice is different from that in adult mice and that the delayed clearance of *Pneumocystis* from the lungs of infant mice is due at least in part to immature dendritic cell function.

If dendritic cell function and the neonatal lung environment do not provide the appropriate stimulatory signals to T cells, it would be expected that even adult T cells in the neonatal lung environment would not respond normally to *Pneumocystis*. To determine whether the neonatal lung environment is inhibitory for developing an immune response to *Pneumocystis*, adult T cells were adoptively transferred into infected neonatal mice. Even when given adult T cells, neonatal mice had a delayed kinetics of cellular infiltration and clearance of *Pneumocystis* compared to reconstituted adult mice [49]. It is clear from these studies that antigen presentation and recruitment of T cells into the lungs is not efficient in neonatal mice. This results in the inability to mount a response to *Pneumocystis* until about the age of weaning [41].

T Lymphocytes

There is a large body of literature suggesting that neonatal T-cell function is immature compared to T-cell function in adults. However, more recent data suggest that there is no intrinsic defect in neonatal T cells but that the dose and mode of antigen challenge can affect the response [65–68]. Upon challenge with

Pneumocystis, there is a delay in the infiltration of CD4⁺ T cells into the lungs of mice infected within 72 hr of birth compared to the rate of infiltration in adults. Adult mice generate an alveolar T-cell response to *Pneumocystis* within 7 days postinfection, whereas it takes a full 3 weeks for infant mice to mount a response (Fig. 1) [41,49,50]. This delay could be due to the inability of T cells to respond to chemotactic signals, the lack of chemotactic signals, the lack of antigen stimulation, or simply low numbers of T cells able to respond. It has been reported that neonatal murine T cells are able to respond to signals through the T-cell receptor (TCR) when appropriate costimulatory signals are available [58]. Notably, T cells from neonates express reduced levels of CD40L in response to various stimuli, and it is known that the CD40-CD40L interaction is required for host defense against *Pneumocystis* [69–72]. This could result in ineffective stimulation of alveolar macrophages, but this has yet to be investigated.

To determine whether T cells isolated from neonatal spleens are able to respond to *Pneumocystis* when provided with appropriate signals, an adoptive transfer strategy was used [50]. T cells from 10-day-old mice were isolated from the spleens and transferred into *Pneumocystis*-infected adult mice with severe combined immunodeficiency (SCID). In this context, the ability of the SCID mice reconstituted with T cells from infants to resolve PcP was not different than that of mice reconstituted with T cells from adults [50]. The kinetics of migration of T cells into the lungs of infected SCID mice was similar between the groups given T cells from infants vs. those from adults [50]. Consistent with this finding, there was no difference in the ability to proliferate or produce IFN- γ when T cells from the draining lymph nodes of 10-day-old and adult mice infected with *Pneumocystis* were stimulated ex vivo with concanavalin A or plate-bound anti-CD3 [50]. These data suggest that T cells from neonatal mice are fully capable of responding to antigen. Furthermore, it suggests that either the frequency of specific cells is so low that it takes 3 weeks to generate enough to mount a detectable response or the lung environment is not conducive to mounting an inflammatory response to *Pneumocystis*.

Notably, most of the data regarding the role of neonatal T cells in host response to *Pneumocystis* has been directed at CD4⁺ cells, since it has been demonstrated that in the absence of these cells, but not CD8⁺ cells, there is no ability to clear the infection [57,73]. However, more recently, it has been shown that in the absence of CD4⁺ cells, CD8⁺ cells offer partial protection against *Pneumocystis* in a murine model of infection [74,75]. In a case report, an infant developed PcP in spite of having normal humoral immunity as well as normal CD4⁺ T cells in the peripheral blood [76]. This infant had a disorder that led to the deletion of CD8⁺ cells from the thymic medulla. Although there are no experimental data in animal models, this suggests that CD8⁺ T cells may have an important role in host defense against *Pneumocystis* in infants, and these cells warrant further scrutiny.

C. Humoral Immunity

A number of studies have indicated that *Pneumocystis*-specific antibody responses have a significant role in host defense against the organisms [77,78]. Furthermore, mice deficient in B cells are susceptible to PcP, suggesting that either specific B cells or antibody are critical for host defense against *Pneumocystis* [79,80]. B cells from neonatal mice have been shown to be deficient in proliferative capacity in response to anti-Ig + IL-4 treatment but not through ligation of CD40 [81]. Consistent with this finding, it has been shown that B cells from neonates are capable of class switching and producing antibody in the presence of appropriate T-cell help [82,83]. Little is known about the B-cell response to *Pneumocystis* in infants; however, over 90% of individuals have circulating *Pneumocystis*-specific antibody by the age of 2, suggesting that effective B-cell responses have occurred at least by that time [29].

Maternal Antibody

Very young infants acquire antibody in utero as well as through suckling, so it is thought that infants are protected from a number of infectious agents as long as they have maternal antibody. Maternal antibody begins to wane in human infants by about 6 months of age. In this regard, it was reported that a foal with hypogammaglobulinia, presumably due to the lack of colostrum from the dam, developed PcP and died at 46 days of age [84]. In a murine model of *Pneumocystis* infection, dams were immunized with the organisms prior to breeding and during gestation in order to develop high *Pneumocystis*-specific antibody levels in both their blood and milk [41]. Offspring of the immunized dams were infected with *Pneumocystis* within 72 hr of birth. Neonatal mice from immunized dams had elevated serum *Pneumocystis*-specific antibody at day 2 postinfection, compared to neonates from naive dams [41]. Elevated antibody levels corresponded to a 90% reduction of lung *Pneumocystis* burden between days 15 and 20 postinfection, whereas there was no reduction on lung burden over that time in mice from naive dams [41]. Even though maternal antibody facilitated expedited clearance of *Pneumocystis*, the response was still slower than that seen in immunized adult mice and was similar to the kinetics of clearance of adult naive mice. Furthermore, the decline in lung organisms corresponded with the onset of the inflammatory response in the lungs and may indicate that maternal antibody did not enter the lungs until an inflammatory response was initiated [41]. However, thereafter, the presence of antibody facilitated clearance of the *Pneumocystis* organisms. Unfortunately, antibody was not measured in the lungs, hence this is speculative. These studies clearly show that maternal antibody can play a role in clearance of *Pneumocystis* in neonates.

B Lymphocytes and Antibody

There are no published reports regarding the role of B cells in host defense to *Pneumocystis* in infants and very little is published for adults. However, it is clear that *Pneumocystis*-specific antibody is important for host defense, since children with X-linked hyper-IgM syndrome having a mutation in the CD40L gene, which results in ineffective T cell-dependent antibody responses, are susceptible to PcP [13,14]. Soulez et al. reported that weanling rabbits are susceptible to infection with *Pneumocystis*, and that when they developed a humoral response, the organisms were cleared [31]. Consistent with this observation, in mice infected with *Pneumocystis* as infants, serum antibodies are very low compared to those in infected adults through about 5 weeks postinfection [41]. Similarly, *Pneumocystis*-specific antibody-secreting cells increased in the draining lymph nodes of adult mice between days 9 and 15 postinfection, whereas these cells were very difficult to detect in mice infected as infants through day 35 postinfection (Fig. 2) (Garvy and Harmsen, unpublished observations). The detection of *Pneumocystis*-specific antibody in mice infected as infants corresponded to clearance of the organisms. Furthermore, this antibody response was protective even when 6-week-old mice infected as neo-

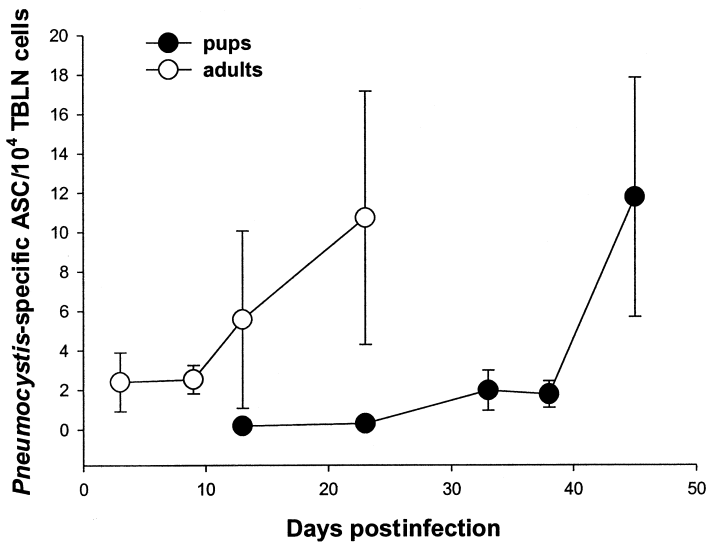


Figure 2 The appearance of *Pneumocystis*-specific antibody secreting cells (ASC) is delayed in the lymph nodes of mice infected as neonates as compared to adults. *Pneumocystis*-specific ELISpot assays were performed using draining lymph node cells from mice infected with *Pneumocystis* within 72 hr of birth or as adults. Data are expressed as frequency of ASC per 10⁴ plated cells and represents the mean $\pm$ SD of four to five mice per group. (B. A. Garvy and A. G. Harmsen, unpublished results.)

nates were depleted of CD4⁺ cells and rechallenged with *Pneumocystis*. These animals had high levels of *Pneumocystis*-specific IgG in their sera and cleared a second challenge with *Pneumocystis* in the absence of T cells (Garvy and Harmsen, unpublished observations). These studies are consistent with the known importance of B cells and antibody for protection against *Pneumocystis* in adults; however, it is still not clear why there is a delay in the appearance of antibody and antibody secreting cells in neonatal mice. One possibility is the lack of appropriate costimulatory signals, such as CD40L expressed on T cells, as discussed above. It is also possible that migration of B cells to the site of infection is delayed. This could be due to the lack of chemokine production or the inability to upregulate adhesion molecules. These issues are currently being examined.

D. Cytokines and Chemokines

A significant role for TNF- α , IL-1, and IFN- γ during resolution of PcP in adult mice has been reported by several investigators and is discussed in depth elsewhere in this volume [74,85–90]. There is a significant reduction in the mRNA expression of TNF- α and IFN- γ in response to *Pneumocystis* infection in neonates compared to adults during the first 2 weeks postinfection (Fig. 3) [50]. This delay in the expres-

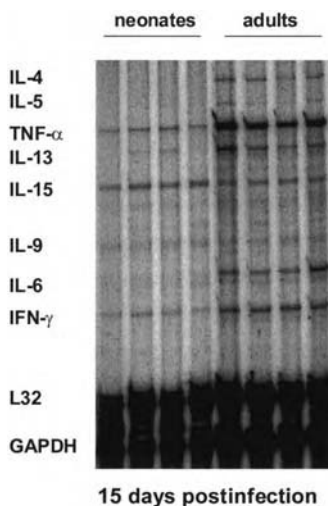


Figure 3 Expression of cytokine mRNA in response to *Pneumocystis* infection is delayed in mice infected as neonates compared to adults. RNase protection assays were used to quantitate mRNA levels at day 15 postinfection. There is reduced expression of several cytokines, including TNF- α and IFN- γ in the neonatal lungs compared to the adult lungs at the time point shown.

sion of proinflammatory cytokines corresponds to the delayed inflammatory response in the alveolar spaces and the delayed clearance of *Pneumocystis* from the lungs of neonatal mice [49,50]. Interestingly, adoptive transfer of adult T lymphocytes into the lungs of neonatal mice did not facilitate the upregulation of cytokine mRNA expression [49]. However, when neonatal T cells were transferred into *Pneumocystis*-infected adult SCID mice, TNF- α and IFN- γ mRNA expression was upregulated with the same kinetics as in mice reconstituted with adult T cells [50]. TNF- α produced primarily by macrophages and T cells is an important proinflammatory cytokine that regulates the expression of adhesion molecules, chemokines, and chemokine receptors and promotes maturation of dendritic cells to APCs [61,62,91]. In this regard, expression of adhesion molecules ICAM-1 and VCAM-1 is decreased in the lungs of mice infected with *Pneumocystis* as neonates compared to those infected as adults (Qureshi, Cook-Mills, and Garvy, unpublished observations). The absence of a TNF- α response could account for the delayed clearance of *Pneumocystis* from the lungs of infant mice; however, it is not known why TNF- α is not produced in response to this infection.

In addition to delayed upregulation of cytokines in response to *Pneumocystis* in neonatal lungs, chemokine mRNA expression is also delayed in them as compared to adults [49]. Reconstitution of *Pneumocystis*-infected infant SCID mice with adult lymphocytes did not induce expression of lymphotactin, RANTES, MIP-1 β , and other chemokines, whereas reconstitution of adult SCID mice did result in increased chemokine mRNA expression [49]. The lack of RANTES, MIP-1 α , MIP-1 β , and MCP-1 could account for the seeming inability of immature dendritic cells in the lungs of neonates to mature to APCs and migrate to draining lymph nodes [61]. In addition, lymphocyte migration into the infected lungs is dependent on chemokines such as MCP-1, so the delayed inflammatory response is consistent with delayed expression of chemokines in the lungs of infant mice [63,92].

IV. Neonatal Lung Environment and PcP

It is evident from the studies described above that the unresponsiveness to *Pneumocystis* can be due to a number of deficiencies in the neonate. Dendritic cell maturation and migration to draining lymph nodes appears to be suppressed, as are cytokine and chemokine responses to *Pneumocystis* in the lungs of neonatal mice. However, this does not mean that there are no stimuli capable of inducing inflammation into the lungs of neonates. Hyperoxia induces increased RANTES, MIP-1 α , and MCP-1 mRNA levels in the lungs of 4-day-old mice [93]. In addition, neonatal mice infected with *Streptococcus pneumoniae* mount a neutrophil response in the lungs that is as intense as that in adult mice [64]. This suggests that *Pneumocystis* does not pose a threat to the survival of neonatal mice, so the immune response is dampened to protect the lungs from potentially dangerous inflammatory responses.

Notably, increasing the dose of *Pneumocystis* used to infect neonatal mice 50-fold did not result in a change in the kinetics of the response, indicating that the suppression of the immune response could not be overridden by increased lung burden of organisms [50]. It is not clear how *Pneumocystis* evades immune surveillance for so long in neonatal lungs. It may be that a critical burden of organisms is necessary to induce some form of injury on the lungs. Neonatal mice are known to be able to survive under conditions of low oxygen tension, so it may take a comparatively larger *Pneumocystis* burden than in adults to trigger an inflammatory response [94].

As suggested above, *Pneumocystis* may be able to evade host surveillance in the lungs of neonates. Alternatively, a significant amount of postnatal development takes place in the lungs and an inflammatory response could be detrimental to this development. Ninety percent of the alveolar spaces that are available in adults are generated over the 2 months after birth in rats and 18 months in humans [54]. It is possible that there is active suppression of inflammatory responses in the lungs of neonates which can be overridden when a dangerous pathogen is present but which suppresses immune responses to fairly innocuous agents and buys time until postnatal development is completed. Notably, alveolar macrophages are known to be immunosuppressive toward T cells, and this may contribute to the lack of an immune response to *Pneumocystis* in neonatal mice [95,96]. In addition, constitutive expression of all three isoforms of TGF- β mRNA was found in the lungs of neonatal mice and may contribute to the unresponsiveness to *Pneumocystis* [50]. TGF- β is critical for postnatal development in the lungs [97]. However, TGF- β also has immunosuppressive actions, so that mice deficient in TGF- β 1 die within 2 weeks of age of a severe lymphoproliferative disease [98]. IL-10 mRNA expression was found in the lungs of neonatal mice, although at lower levels than in adults [50]. In the absence of IFN- γ , even small amounts of IL-10 may contribute to an immunosuppressive environment; this warrants further investigation. Along these same lines, suppressor of cytokine signaling-1 (SOCS-1) mRNA expression was found in the lungs of neonatal mice whether they were infected with *Pneumocystis* or not (Young and Garvy, unpublished observations). SOCS-1 is a known suppressor of IFN- γ gene expression and so may also contribute to an environment that is immunosuppressive [99]. Although a case can be made for the possibility that the neonatal lung environment is immunosuppressive, definitive experiments need to be performed before this issue can be resolved.

V. Conclusion

A number of immunodeficiency states predispose young children and infant animals to *Pneumocystis* infection. However, it has recently become evident that immunocompetent children and infant animals can carry *Pneumocystis* infection without obvious signs of illness, and that around the time of weaning an immune response is initiated that results in clearance of the organisms. Studies using neo-

natal mice as a model for infection have indicated that there is a significant delay in the cytokine and chemokine response to *Pneumocystis*. Maturation and migration of dendritic cells to the draining lymph nodes is inefficient in neonatal mice, likely resulting in delayed stimulation of antigen-specific T cells. Migration of T cells into the alveolar spaces is delayed in neonates compared to adults. In addition, the humoral response to *Pneumocystis* is delayed in neonates. This delay in the immune response to *Pneumocystis* in infants results in a permissive environment for growth of the organisms. This raises the question of whether the seeming inability to respond to *Pneumocystis* is due to “immaturity” in the immune system, particularly good evasion properties of the organisms, or some sort of active suppression in the lungs of neonates. We currently favor the hypothesis that delayed clearance of *Pneumocystis* in the lungs of neonatal mice compared to adults is due largely to suppression of immune responses in postnatally developing lungs. However, a significant amount of experimental work will be required to confirm this hypothesis.

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9

Interactions of *Pneumocystis* with Alveolar Macrophages and Epithelial Cells

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I. Introduction

Pneumocystis carinii and related species survive as obligate or nearly obligate intraalveolar fungal parasites. Definitive evidence of continuous proliferation outside the confines of the lower respiratory tract has not yet been documented. Additional studies reveal that interactions of *Pneumocystis* with specific resident cells of the lung mediate important regulatory functions in the life cycle of this organism. In addition, host-cell recognition of the *Pneumocystis* surface is an important event in the initiation of inflammatory systems and host defense responses during the development of this pneumonia.

Pneumocystis organisms have been reported to interact with a variety of cell types present in the lower respiratory system, including lymphocytes and natural killer cells, alveolar macrophages, neutrophils, and alveolar epithelial cells [1–6]. Additional evidence suggests that *Pneumocystis* may also colonize epithelium of the upper and conducting respiratory tracts including the oropharynx and major airways [7,8]. The central interactions of *Pneumocystis* with lymphocytes, particularly CD4 cells, are essential to host defense against this infection and are extensively reviewed in Chapter 9 of this volume. The present chapter focuses on the mechanisms and consequences of *Pneumocystis* interactions with

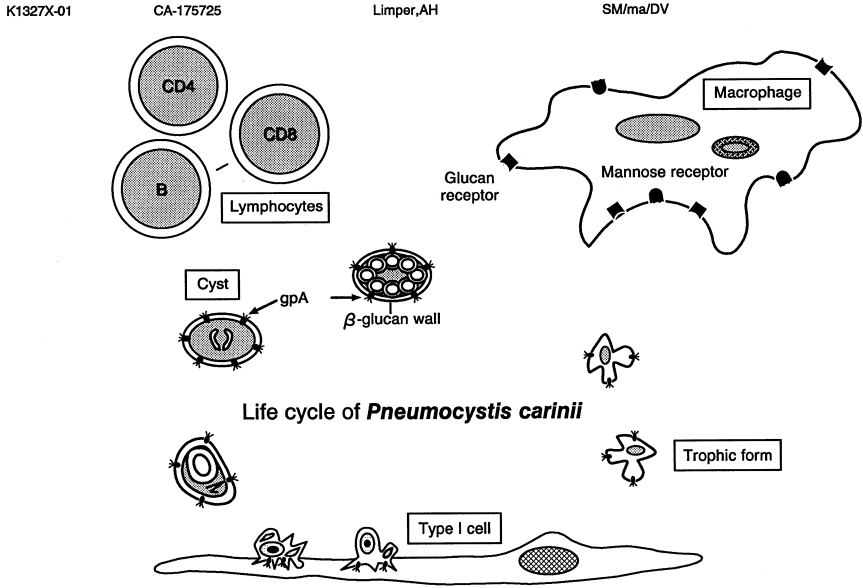


Figure 1 Interaction of *Pneumocystis* with cells of the lower respiratory tract. Recognition of *Pneumocystis* by CD4 + T cells is essential for clearance of infection and institution of immunological memory. Alveolar macrophages are stimulated by lymphocyte-derived IFN- γ to phagocytize and degrade in *Pneumocystis*. Macrophages are further stimulated by β -glucans on the surface of the organisms to release TNF- α , reactive oxidants and eicosanoid metabolites, which further promote the inflammatory state of the lung, including the recruitment of neutrophils that mediate injury to the alveolar capillary units. (Adapted from Ref. 33.)

alveolar macrophages and respiratory epithelial cells (Fig. 1). Macrophages serve as important front-line cells, which mediate host recognition of the organism, initiation of inflammatory responses, and clearance of this infection [9,10]. In contrast, interactions of *Pneumocystis* with alveolar epithelial cells appear to have important consequences on repair of the host epithelial lining as well as on modulation of inflammation and proliferation of this opportunistic fungus [6,11–13].

A. Mechanisms of Interaction of *Pneumocystis* with Alveolar Macrophages

Alveolar macrophages represent the principal phagocytic cells of the lower respiratory tract, which mediate initial uptake and degradation of invading microorga-

nisms such as *Pneumocystis* [9,14,15]. The precise mechanism by which host macrophages recognize and internalize *Pneumocystis* has been the subject of intensive investigation. Evolving evidence indicates that a complex variety of receptors mediate these interactions. Of greater significance is the fact that specific receptor-ligand interactions initiate divergent activation responses ranging from uptake and degradation of the fungus to inflammatory activation of the phagocyte, with release of TNF α , oxidants, chemokines, and other potent mediators (Table 1) [4].

In the absence of protein opsonins contained in epithelial lining fluid and serum, the uptake of *Pneumocystis* by alveolar macrophages is principally mediated by macrophage mannose receptors [4,16,17]. The macrophage mannose receptor is a pattern-recognition molecule that represents an important innate clearance mechanism in immunocompetent hosts [16,18–20]. Ezekowitz and col-

Table 1 Host Protein and Receptor Interactions with *Pneumocystis*

Host protein/receptor	<i>Pneumocystis</i> ligand(s)	Activity	Refs.
Mannose receptors	gpA/msg	Mediates macrophage uptake.	17,20
Glucan receptors	β -glucan	Mediate macrophage uptake and inflammatory activation.	4,10
Fibronectin	gpA/msg β -glucan	Augments binding to macrophages and epithelial cells. Increases macrophage release of TNF- α .	87,61
Vitronectin	β -glucan	Augments binding to epithelial cells. Increases macrophage release of TNF- α .	5,61
Immunoglobulin	Unknown and multiple	Augments phagocytosis Enhances macrophage release of eicosanoids.	133–135
Surfactant protein-A	gpA/msg	Augments binding to macrophages. Effect on phagocytosis controversial.	107–112
Surfactant protein-D	gpA/msg β -glucan	Augments binding to macrophages but decreases phagocytosis. Decreases macrophage release of TNF- α .	34–115
Glycosphingolipids (lactosylceramide)	β -glucan	Mediates inflammatory activation of epithelial cells.	95

leagues demonstrated that soluble yeast mannan competitively inhibits uptake of rat *Pneumocystis* by alveolar macrophages [17]. Furthermore, transfection of CHO cell with mannose receptor constructs successfully conferred the ability of these cells to phagocytize *Pneumocystis* [17]. O’Riordan and coworkers subsequently confirmed that the mannose-rich major glycoprotein complex, termed msg or gpA, on the surface of rat-derived *Pneumocystis* was the target ligand recognized by the macrophage mannose receptor pathway [20]. The addition of solubilized gpA resulted in competitive concentration-dependent inhibition of rat *Pneumocystis* attachment to alveolar macrophages in culture, thus indicating that the interaction between *Pneumocystis* gpA (msg) and the macrophage mannose receptor was essential for binding and uptake of *Pneumocystis* by the phagocyte [20].

In addition to interactions of the gpA surface complex of *Pneumocystis* with mannose receptors, other receptor-ligand interactions also provide potent activity in the interactions of *Pneumocystis* with alveolar macrophages. Rat-derived *Pneumocystis* cell wall also contains abundant β -glucans and chitins [21]. Emerging evidence indicates that β -glucans further represent a significant mechanism by which macrophages recognize *Pneumocystis* [4,10]. Fungal β -glucans are branched glucose homopolymers, arranged with β -1,3 linkages, containing variable amounts and patterns of β -1,6 branched side chains. β -glucans represent major structural components of many fungal cell walls and are abundant in the cystic forms of *Pneumocystis* [10,22–24]. Additional evidence suggests that lower level expression of these molecules is also present in the walls of *Pneumocystis* trophic forms [24,25]. In experiments analogous to the mannose receptor studies, Hoffman and colleagues demonstrated that yeast β -glucan in sufficient concentrations could also competitively inhibit binding and uptake of rat-derived *Pneumocystis* by cultured macrophages [4]. These observations are particularly interesting, since β -glucans interact with macrophage receptors, which are distinct from mannose receptors [26]. The nature of the macrophage receptor(s) interacting with *Pneumocystis* continues to be intensively studied. Recent evidence indicates that fungal β -glucans can bind a variety of cell-surface receptors, including the CD11b/CD18 integrin receptor (Mac-1), the newly discovered dectin-1 receptor found on dendritic cells and macrophages, as well as a putative nonintegrin receptor present on monocytoid cells [27–31]. Recent studies propose that the TLR 2 (toll-like receptor 2) may also interact with fungal zymosan, a cell wall product comprising predominantly β -glucans and mannans [32].

Controlled culture experiments imply simple interactions of *Pneumocystis* with alveolar macrophages; in the alveolar spaces, however, the organisms are bathed in protein-rich epithelial lining fluid containing both lung-specific and serum proteins. Indeed, several host proteins such as immunoglobulins, vitronectin, fibronectin, and surfactant proteins bind tightly to *Pneumocystis* and further

facilitate interactions of the organisms with alveolar macrophages (Table 1) [33–38]. These observations further underscore the complex nature of the recognition, binding, and response of alveolar macrophages to *Pneumocystis*. The modulating effects of these host proteins are discussed later in this chapter.

II. Consequences of *Pneumocystis* Interactions with Alveolar Macrophages

Ultrastructural and laboratory studies reveal that alveolar macrophages bind and internalize rat-derived *Pneumocystis* organisms in a time-dependent fashion, with optimal uptake requiring 4 to 8 hr [9,14,15,39]. Following attachment to the alveolar macrophage, *Pneumocystis* organisms are internalized, forming phagolysosomes, and are subsequently degraded [9,14,15]. Although several lines of evidence using in vitro systems indicate that alveolar macrophages bind, internalize, and degrade *Pneumocystis*, direct evidence supporting the role of intact macrophage function in the clearance of *Pneumocystis* infection from the host has only recently emerged.

It is well established that decreased lymphocyte function predisposes to *Pneumocystis* pneumonia. However, additional evidence indicates that reduced macrophage function can also promote the development of this infection. Alveolar macrophages from individuals with AIDS demonstrate marked inhibition of mannose receptor surface expression when compared to macrophages obtained from healthy hosts [40]. Reduced macrophage mannose receptor expression results in significantly impaired binding and phagocytosis of *Pneumocystis* organisms [40,41]. Alveolar macrophage dysfunction also occurs in other immunosuppressed individuals who have a predisposition to develop *Pneumocystis* pneumonia, including bone marrow transplant recipients, patients undergoing prolonged immune suppression, and individuals with advanced malignancy treated with chemotherapeutic agents [42–45].

Studies of rat-derived *Pneumocystis* cultured with normal alveolar macrophages document digestion of the organisms over time [9]. To formally determine the role of macrophages in the clearance of *Pneumocystis* infection, Limper and colleagues tested the ability of alveolar macrophage-depleted rats to remove rat-derived *Pneumocystis* organisms from the lower respiratory tract [9]. Alveolar macrophages were depleted from the lungs of normal rats by administering intratracheal liposomal biphosphonate, an agent that causes apoptosis of macrophages in the lung [46]. While control rats treated with sham liposomes (liposomes without biphosphonate) eliminated most of the inoculated *Pneumocystis* organisms, macrophage-depleted rats demonstrated strikingly impaired clearance of *Pneumocystis*, indicating that alveolar macrophages have a central role in the uptake and clearance of *Pneumocystis* infection in the normal host [9]. Taken

together, these studies indicate that alveolar macrophages provide essential clearance functions during *Pneumocystis* pneumonia in the intact host.

III. Macrophage Inflammatory Responses to *Pneumocystis*

During the course of *Pneumocystis* pneumonia, a variety of mediators are generated by alveolar macrophages, including reactive oxidant species, eicosanoid metabolites, and cytokines and chemokines, which potentiate the inflammatory responses toward this pathogen [47–50]. Although the specific roles of these inflammatory mediators are still under investigation, such responses promote eradication of *Pneumocystis* pneumonia by the host. Recent studies also suggests that while host inflammatory systems are necessary for the elimination of *Pneumocystis* infection, these responses may also lead to pulmonary injury, predisposing the host to alveolar edema formation and respiratory impairment [51–53]. For this reason, an understanding host inflammatory responses to *Pneumocystis* is of critical importance and may have provide insights into clinical features of *Pneumocystis* pneumonia.

Several macrophage-derived cytokines have been clearly implicated in the pathogenesis of *Pneumocystis* pneumonia. When stimulated with *Pneumocystis*, alveolar macrophages release tumor necrosis factor alpha (TNF- α), interleukin 1-beta (IL-1 β), interleukin 8 (IL-8), interleukin 10 (IL-10) and macrophage inflammatory protein 2 (MIP-2) [4,10,50,54–56]. Among these, TNF- α exerts particularly essential activity necessary for the clearance of this infection and the initiation of concomitant lung inflammation [49]. Treatment of *Pneumocystis*-infected rodents with neutralizing antibodies to TNF- α results in delayed clearance and increased severity of pneumonia [57]. Additional strong evidence indicates that treatment of *Pneumocystis*-challenged immunocompetent mice with an adenovirus encoding a TNF inhibitor gene delays clearance and enhances chronicity of *Pneumocystis* infection [58]. Alveolar macrophage production of TNF- α has been shown to be triggered by β -glucan, present in the *Pneumocystis* cell wall, interacting with cognate receptors on the phagocyte [4,10,59]. This stimulatory activity of *Pneumocystis* β -glucan is enhanced by its interactions with both vitronectin and fibronectin present within the alveolar lining fluid [36,60,61].

We have further demonstrated that TNF- α released by alveolar macrophages is induced by a cell wall fraction of rat-derived *Pneumocystis* that is composed virtually entirely of carbohydrates [10]. Furthermore, macrophage TNF- α response to this carbohydrate fraction was significantly diminished by β -glucanase digestion, thus confirming that these glucan components represent potent stimulants of TNF- α release by alveolar macrophages challenged with *Pneumocystis* [10,59]. Other components of the *Pneumocystis* cell wall, such as the gpA major surface glycoprotein, may also stimulate macrophages to liberate

TNF- α [50]. However, direct comparisons of the macrophage stimulatory activity of β -glucans and gpA seem to favor glucans as being far more potent [4,10]. Indeed, neutralization of macrophage mannose receptors, which mediate gpA binding, has little if any effect on macrophage release of TNF- α in response to either intact rat *Pneumocystis* organisms or isolated *Pneumocystis* cell wall fractions [4,10].

Although essential for clearance of *Pneumocystis* infection, the mechanisms by which TNF- α facilitates elimination of this organism remain poorly defined. TNF- α promotes the upregulation of cell-surface adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), which enhances the recruitment of inflammatory cells including neutrophils, monocytes, and lymphocytes in the lung [62]. *Pneumocystis* infection in rats is accompanied by robust expression of ICAM-1 on epithelial cells as well as on the vascular endothelium. The expression of ICAM-1 appears to be mediated both by direct contact of the organisms with the epithelium and indirectly through *Pneumocystis*-induced release of TNF- α from macrophages [62]. ICAM-1 expression promotes the phagocytic cell recruitment necessary for clearance of organisms [63,64]. In addition, TNF- α also activates local inflammatory airway cells and alveolar epithelial cells to secrete other cytokines, chemokines, and inflammatory mediators, further enhancing the inflammatory response at the site of *Pneumocystis* infection. Finally, TNF- α has been shown to bind to rat-derived *Pneumocystis* (probably to the β -glucan component of the cell wall) and perhaps may exert a static effect on organism viability [65]. Other investigations have not verified such an effect on viability. Therefore the direct effects of TNF- α on *Pneumocystis* remain uncertain at this time.

It is likely that the increased susceptibility to *Pneumocystis* pneumonia associated with low CD4 lymphocyte counts is partly related to lack of IFN- γ as part of the normal host response to *Pneumocystis*. In vitro studies demonstrate that although not directly lethal to *Pneumocystis*, IFN- γ has an important role in the activation of alveolar macrophage responses to *Pneumocystis*. Mechanisms by which IFN- γ facilitates host defenses include upregulation of TNF- α production, increased generation of superoxide species, and increased release of reactive nitrogen intermediates [65–67]. In addition to activation of alveolar macrophages, IFN- γ also participates in host defense by decreasing alveolar epithelial cell integrin expression, resulting in decreased attachment of *Pneumocystis* to the host epithelium [68]. The importance of IFN- γ in host responses to *Pneumocystis* is also supported by several in vivo studies [69–72]. Administration of gamma interferon by aerosol to rodents with *Pneumocystis* infection reduces the intensity of infection, despite persistent CD4 + lymphocyte depletion in these animals [66]. In contrast, one study failed to demonstrate a significant effect on *Pneumocystis* infection following inhibition of IFN- γ with neutralizing antibodies [69]. Similarly, Rudmann and coworkers recently showed that deficiency of IFN- γ receptors

alone in mice does not suppress clearance of *Pneumocystis* infection [72]. However, simultaneous deletion of IFN- γ and TNF- α receptor genes in mice results in markedly increased susceptibility to *Pneumocystis* [72]. These data demonstrate that IFN- γ participates in host defense against *Pneumocystis*, but further evaluation will be needed to define its overall contribution. Whether treatment with IFN- γ has any role in the management of human *Pneumocystis* pneumonia will similarly require further investigation.

In addition to TNF- α and cytokine production, stimulation of alveolar macrophages with *Pneumocystis* causes release of reactive oxidants, including hydrogen peroxide. Macrophage oxidant generation in response to rat-derived *Pneumocystis* is also mediated in part through interaction of β -glucan components of *Pneumocystis* with glucan receptors present on alveolar macrophages [47]. Macrophages are further stimulated to generate inflammatory eicosanoids such as prostaglandin E2 when challenged with rat *Pneumocystis* [48]. The induction of reactive oxidants and eicosanoids contributes to the inflammatory response, although the specific role of these mediators in clearing *Pneumocystis* infection has not yet been determined.

Although macrophage inflammatory responses elicited by *Pneumocystis* are required for elimination of infection, excessive inflammation may have deleterious effects on the host. In particular, exuberant TNF- α , IL-1, and IL-8 responses increase endothelial permeability, enhance alveolar edema, and promote marked neutrophilic infiltration into the lungs [51,53,73,74]. The activity of neutrophils in host defense against *Pneumocystis* has not yet been well defined. However, it is well established that exuberant neutrophilic lung infiltration potentiates lung injury and contributes to respiratory impairment during this infection. Accordingly, increased neutrophil lung recruitment has been correlated with increased mortality and the development of worse lung injury during *Pneumocystis* pneumonia [52,75–77]. Indeed, respiratory impairment and death correlate more closely with the extent of lung inflammation than with the burden of organisms during *Pneumocystis* pneumonia in humans [52]. This theory is supported by the clinical observation that severe *Pneumocystis* pneumonia responds favorably to the institution of anti-inflammatory agents such as corticosteroid therapy given adjunctively with antimicrobial drugs [78–81].

Increased understanding of the role of host inflammation may provide insight to the clinical observation that *Pneumocystis* pneumonia in the non-HIV patient tends to be associated with significantly greater adverse respiratory outcomes than *Pneumocystis* pneumonia in the HIV population [52,82]. One may speculate that the pervasive and profound immunodeficiency that accompanies the later stages of HIV infection provides a milieu that enables proliferation of the parasite in the human host without excessive local injury to the host lung. In contrast, *Pneumocystis* pneumonia occurring in the non-HIV setting (generally less profound immune deficiency than that seen in HIV) is associated with greater

local inflammatory responses in the lung and significantly enhanced morbidity and mortality [52].

IV. Mechanisms of *Pneumocystis* Interactions with Alveolar Epithelial Cells

Several important interactions occur between *Pneumocystis* and the alveolar epithelial lining of the host. Ultrastructural studies indicate that *Pneumocystis* trophic forms adhere tightly predominantly to type I alveolar epithelial cells (Fig. 2) [12,83–85]. This attachment process is strongly facilitated through interactions of *Pneumocystis* with adhesive glycoproteins that are present in increased quantities in the lower respiratory tract during *Pneumocystis* pneumonia (Table 1). Several studies indicate that vitronectin and fibronectin bind to *Pneumocystis* and augment attachment of the organisms to epithelial cells through corresponding integrin receptors on host type I alveolar cells [86,87]. Fibrinogen may be another

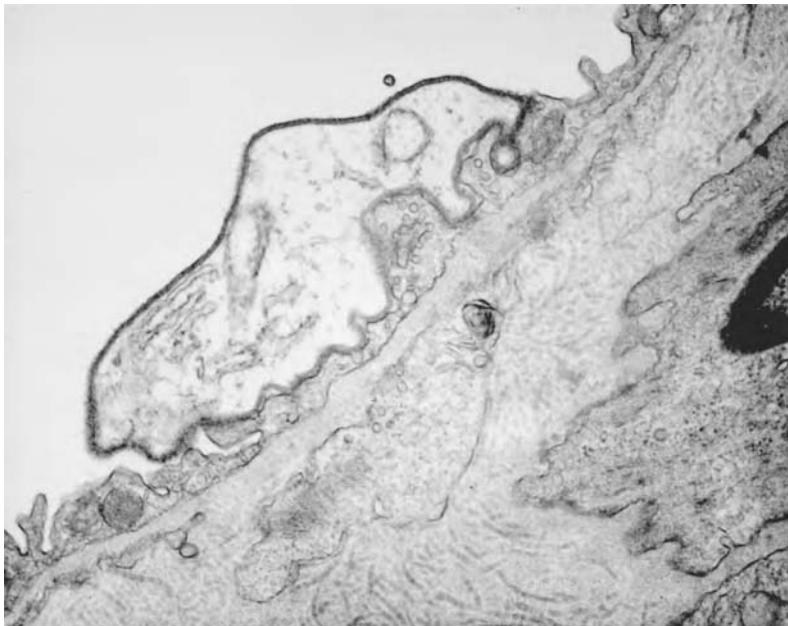


Figure 2 Transmission electron micrograph demonstrating in *Pneumocystis* trophic forms adhering to the alveolar epithelium. Rat lung with *Pneumocystis* pneumonia was examined using electron microscopy. *Pneumocystis* trophic forms (1 to 2 μ M in greatest diameter) adhere to alveolar epithelium with interdigitation of their membranes with the membranes of host cells. (Magnification $\times 12,000$). (From Ref. 12.)

glycoprotein participating in the attachment of trophic forms to alveolar epithelial cells [88]. *Pneumocystis* organisms exhibit a lesser extent of attachment to type II cells, possibly due to differences in surface integrin expression [89]. Additional investigations suggest that type II epithelial cells may also exert a directly toxic effect on *Pneumocystis* organisms [90].

Stable attachment of *Pneumocystis* to alveolar epithelial cells is associated with cytoskeletal rearrangement by the organisms. Electron microscopic studies demonstrate that *Pneumocystis* trophic forms interdigitate their cell membranes with those of the alveolar epithelial lining [84,85]. During the attachment process, trophic forms extrude filopodial extensions onto the cell surface. Cultures of purified rat-derived *Pneumocystis* on alveolar epithelial cells suggest that stable association of the organisms with the epithelium is maximal after 4 to 6 hr of coinubation [6]. However, the kinetics of filopodial extrusion are not yet well established. The membranes of the host and organism become intimately associated, but true membrane fusion and engulfment of the organism by host epithelial cells generally does not occur. Accordingly, cytoskeletal toxins such as colchicines and cytochalasin B have been shown to significantly reduce attachment of rat-derived *Pneumocystis* to alveolar epithelial cells in culture [6]. Furthermore, agents such as benzimidazoles, which affect the organism's cytoskeletal function, have also been proposed to have potential activity against *Pneumocystis* infection [91].

V. Consequences of *Pneumocystis* Interactions with the Alveolar Epithelium

Binding of *Pneumocystis* organisms exerts significant effects on alveolar epithelial cell function. Attachment of rat *Pneumocystis* to A549 cells (a human alveolar epithelial cell line) or mink lung epithelial cells is associated with inhibition of cell growth and replication of the cultured epithelial cells [6,11]. This inhibitory effect on lung cell growth requires direct organisms contact with the host cell, which induces a selective inactivation of the Cdc2 cyclin dependent kinase necessary for host cell cycle progression [11]. Indeed, total epithelial cell Cdc2 protein levels are preserved, yet its phosphorylation state is altered to an inactive form [11]. Impairment of lung epithelial cell growth may in part explain the sluggish repair of lung injury, which often clinically accompanies *Pneumocystis* pneumonia.

Electron microscopic studies of lung specimens obtained from patients and animals with *Pneumocystis* pneumonia also reveal vacuolization and erosion of Type I epithelial cells in association with apposition of the trophic forms of the organisms to lung cells [83]. Ultrastructural studies demonstrate that after seven weeks of *Pneumocystis* pneumonia in the corticosteroid treated rats, Type I pneumocytes reveal degenerative changes and exhibit substantially enhanced perme-

ability to circulating proteins [92,93]. Whether, this epithelial injury is a direct consequence of organisms binding or is instead promoted by associated inflammatory cell products is a matter of controversy. Studies have failed to show any necrotic or apoptotic death of epithelial cells cultured with rat-derived *Pneumocystis*. In addition, epithelial cells cultured with *Pneumocystis* did not reveal any reduction of transmembrane potential induced by the organism [94]. Indeed, epithelial cell transmembrane electrical potentials were increased when *Pneumocystis* organisms were adherent to their surfaces [94]. Thus, the binding of *Pneumocystis* to the alveolar surface may retard repair of lung injury, but the binding itself does not appear to induce substantial direct cell cytotoxicity on the epithelium.

Additional investigations reveal that alveolar epithelial cells release significant quantities of inflammatory cytokines and chemokines following interaction with surface components of *Pneumocystis*, including IL-6, IL-8, and macrophage inflammatory protein 2 (MIP-2) [95–98]. Recent studies indicate that epithelial release of neutrophil-attracting chemokines such as MIP-2 in response to rat-derived *Pneumocystis* may be of a magnitude equal or greater than the level of MIP-2 derived from macrophages challenged with *Pneumocystis* [95]. The attachment of *Pneumocystis* to epithelial cells also induces expression of IL-6, which further promotes expression of integrin receptors on the surface of lung epithelial cells [96,97]. It has been proposed that *Pneumocystis* induction of integrins may thereby promote enhanced attachment of the organisms to the lung epithelial lining. These studies indicate that the relationship between *Pneumocystis* and epithelial cells exerts important functional activities in the establishment of pneumonia.

VI. The Role of Alveolar Proteins in Modulating *Pneumocystis* Interactions with Cells of the Lower Respiratory Tract

A. Fibronectin and Vitronectin

Increased amounts of the serum glycoproteins vitronectin and fibronectin accumulate in the lower respiratory tract during the development of *Pneumocystis* pneumonia [33,36]. Several studies have shown that these glycoproteins interact with a variety of microbes and have active roles in mediating interactions between infectious pathogens and host cells. Both fibronectin and vitronectin bind to rat *Pneumocystis* and mediate attachment to respiratory epithelial cells and alveolar macrophages through corresponding integrin receptors [33]. The binding of fibronectin to rat *Pneumocystis* occurs through the arginine-glycine-aspartate cell-attachment site of fibronectin, whereas vitronectin binds to rat *Pneumocystis* by a predominantly electrostatic mechanism using the heparin-binding domain of

this adhesive glycoprotein [35,38]. In addition to mediating attachment of *Pneumocystis* to lung epithelium, vitronectin and fibronectin also enhance alveolar macrophage activation and release of TNF- α in response to *Pneumocystis* [36]. Vitronectin and fibronectin accumulate in the lung both through local synthesis and via leakage of plasma from injured alveolar capillary membranes. Enhanced tissue expression of these glycoproteins is also enhanced through the induction of abundant circulating levels of IL-6 during *Pneumocystis* pneumonia [61]. Another acute-phase response protein that is thought to be involved in host responses to *Pneumocystis* is fibrinogen [88]. An increase in local synthesis of fibrinogen by lung epithelial cells and locally increased levels of fibrinogen have been observed during *Pneumocystis* infection [88]. The functional significance of elevated levels of fibrinogen in this setting is currently under investigation.

B. Components of Pulmonary Surfactant

Severe *Pneumocystis* pneumonia is further characterized by frothy proteinaceous exudates that fill the alveolar spaces and contain abundant organisms. These frothy exudates can be so predominant as to mimic pulmonary alveolar proteinosis in some patients with *Pneumocystis* pneumonia [99]. Pulmonary surfactant is a complex lipid-protein mixture normally containing 90% lipids and 10% proteins; it is synthesized by the type II alveolar epithelial cell [100,101]. Surfactant lipids are largely responsible for reduction of alveolar surface tension and maintenance of alveolar patency. During severe *Pneumocystis* pneumonia, the normal content of alveolar surfactant is markedly altered, with abundant accumulation of certain surfactant-associated proteins, particularly surfactant protein A (SP-A) and surfactant protein D (SP-D), with associated reductions in surfactant lipid components [34,102–104].

SP-A and SP-D are structurally homologous proteins belonging to C-type family of collagenous, calcium-dependent lectin molecules [100]. In addition to SP-A and SP-D, *Pneumocystis* also interacts with surfactant protein B (SP-B), a smaller lipophilic surfactant protein that is believed to have major activity in conferring the surface tension-reducing properties of surfactant phospholipids [105]. Evidence from several sources suggests that host surfactant proteins contribute to defense mechanisms of the lung against a variety of infective pathogens [100,106]. Both SP-A and SP-D bind to rat *Pneumocystis* and play important roles in modulating the local response to *Pneumocystis* within the lung [34,107]. In addition to modulating host immune responses to *Pneumocystis*, host surfactant abnormalities may also participate in the pathogenesis of hypoxia associated with *Pneumocystis* infection [108,109].

Under basal conditions, SP-A is the predominant surfactant-associated protein present in the lower respiratory tract [110]. Although SP-A levels are elevated in the lavage fluid of patients and rodents with *Pneumocystis* pneumonia, the

precise role of SP-A in the pathogenesis of *Pneumocystis* infection is not well understood [103,104]. SP-A has been proposed to function as a bridging ligand mediating interactions between *Pneumocystis* and alveolar macrophages. SP-A binds the major surface glycoprotein complex (variously termed “msg” or “gpA”) present on the *Pneumocystis* cell wall through its carbohydrate recognition domain [111]. In turn, SP-A coated organisms can interact with collection receptors present on alveolar macrophages [100]. The impact of SP-A–*Pneumocystis* interactions remains unclear. In one set of investigations, Williams and coworkers have shown that SP-A enhances attachment and subsequent uptake of rat *Pneumocystis* by alveolar macrophages [107]. In contrast, Koziel and coworkers reported that the presence of SP-A on the surface of rat *Pneumocystis* actually reduces binding and phagocytosis of *Pneumocystis* by alveolar macrophages [112]. Furthermore, the ability of macrophages to bind *Pneumocystis* correlated inversely with the amount of SP-A present on the surface of the organisms [112]. These contrasting findings may reflect different methodologies used to measure organisms attachment and phagocytosis as well as differences in incubation conditions used to study the kinetics of binding. The effects SP-A interactions with alveolar epithelial cells have not yet be investigated. Recent studies using immune suppressed SP-A knockout mice indicate that mice lacking this collectin have an increased susceptibility to this infection compared to wild-type control mice [113]. Thus, the predominance of evidence suggests that the interactions of SP-A with the host favors host defense against this infection.

In contrast to SP-A, under basal conditions, SP-D represents a relatively minor component of the alveolar lining material. However, during *Pneumocystis* infection in the rat, mouse, and human, the local concentrations of SP-D present in the lower respiratory tract dramatically increase, to the extent that it makes up a significant component of the alveolar exudates [34,114,115]. SP-D also interacts with *Pneumocystis* by binding the gpA surface complex, again utilizing the carbohydrate recognition domain of the collectin [34,116]. In contrast to SP-A, the net effect of SP-D binding to rat *Pneumocystis* appears to favor the organism during establishment of infection [115]. Although SP-D clearly facilitates attachment of rat *Pneumocystis* to the surface of macrophages, phagocytosis of *Pneumocystis* is actually impaired by SP-D [115]. The mechanisms of SP-D’s inhibition of organism uptake appears to be related to the formation of large aggregates of *Pneumocystis* organisms that are too his for effective engulfment by alveolar macrophages [115]. An additional intriguing observation is that TNF- α release by alveolar macrophages exposed to rat *Pneumocystis* is strongly reduced in the presence of SP-D [117]. These data illustrate the complexity of interactions between host proteins and *Pneumocystis*. Rather than being beneficial to the host, these studies reveal that SP-D likely facilitates survival of *Pneumocystis* in the lung by reducing the intensity of the host macrophage immune response and reducing uptake of *Pneumocystis* by alveolar macrophages.

Definitive studies employing the use of SP-D knockout and overexpressing mice may help to further define the role of SP-D in host defense against *Pneumocystis*. Additional studies will also be needed to address the complex interplay of SP-A and SP-D during the development of *Pneumocystis* pneumonia.

In addition to the alterations in SP-A and SP-D expression, *Pneumocystis* also alters SP-B content present in the lung during *Pneumocystis* infection [105]. Lymphocyte-depleted mice with *Pneumocystis* pneumonia exhibit significant deficiency of SP-B [105]. The mechanism by which *Pneumocystis* induces SP-B deficiency in the lung is not entirely clear, but it may also result from the direct interactions of *Pneumocystis* organisms with type II alveolar epithelial cells. It is noteworthy that the suppressive effect of *Pneumocystis* on SP-B secretion is specific, since the concomitant expression of SP-A by lung epithelial cells was unaltered in these investigations [105]. These findings further indicate that the reduction in SP-B secretion was not simply the result of diffuse alveolar epithelial damage. Acquired SP-B deficiency during the course of *Pneumocystis* pneumonia may have important physiologic consequences to the host, since this hydrophobic surfactant associated protein is essential for normal surfactant spreading and preserved lung function. Inherited or acquired deficiencies of SP-B are associated with profound refractory hypoxemic respiratory failure, which is completely unresponsive to replacement of surfactant lipids [118–124]. Animal studies further demonstrate that antibody neutralization of SP-B function also causes abnormalities in gas exchange [119]. Therefore the relative deficiency in SP-B, induced during the development of *Pneumocystis* pneumonia, substantially compounds the impairment of respiratory function.

Furthermore, there is also profound reduction in surfactant lipid content and activity during *Pneumocystis* pneumonia [108,125,126]. Classical studies by Stokes and colleagues have demonstrated that animals with *Pneumocystis* pneumonia exhibit significantly decreased pulmonary compliance, related to decreased pulmonary surfactant [127]. This net reduction in surfactant lipid content is likely mediated through several mechanisms, including enhanced degradation, diminished de novo synthesis, and altered uptake of surfactant by rat-derived *Pneumocystis* organisms [90,105]. Rice and coworkers demonstrated that alveolar type II cells isolated from rats infected with *Pneumocystis* have less basal capacity to synthesize and secrete phosphatidyl choline as well as decreased surfactant release in response to secretagogues [128]. A more complete understanding of the mechanisms and degrees of surfactant abnormalities may provide new insights for novel therapeutic strategies in the management of advanced *Pneumocystis* pneumonia. Several studies have already documented improvement in hypoxemia and respiratory function following administration of exogenous surfactant lipid components to rats [129,130]. In addition, anecdotal reports in infants with *Pneumocystis* pneumonia indicate that surfactant replacement therapy may be beneficial [131,132]. Although promising, these encouraging results are preliminary

and will require validation in larger prospective clinical trials. In addition, further studies are required to evaluate the extent and consequences of surfactant protein abnormalities in humans with *Pneumocystis* pneumonia.

C. *Pneumocystis* Interactions with Additional Molecules Present in the Lower Respiratory Tract During Pneumonia

In addition to local accumulation of surfactant proteins, *Pneumocystis* pneumonia is associated with the accumulation of numerous additional host molecules including serum glycoproteins, immunoglobulins, glycosphingolipids, complement proteins, and other secreted products of immune cells [33]. Accumulation of protein in the airways is the result of both leakage of circulatory proteins from plasma and local synthesis by alveolar resident cells in response to *Pneumocystis* pneumonia. Irrespective of the source, accumulating evidence indicates that these proteins participate in important host-organism interactions during *Pneumocystis* pneumonia. Immunoglobulins are of particular importance, since immunoglobulin expression is necessary for optimal clearance of *Pneumocystis*, presumptively through opsonization of the organisms for rapid and effective phagocytosis by alveolar macrophages [133–135]. In addition, coating of *Pneumocystis* with immunoglobulin augments macrophage inflammatory responses to the organisms [36]. Additional recent studies suggest that interactions of lung glycosphingolipids with *Pneumocystis* can also strongly mediate inflammatory signaling in response to *Pneumocystis* cell wall β -glucans [95,136].

VII. Summary

Although it has been widely accepted that CD4 lymphocytes are the cornerstone of host defense against *Pneumocystis* pneumonia, substantial work further indicates that alveolar macrophages contribute essential functions to host defense during this infection. Indeed, abnormalities in lymphocyte number and function combined with suppression of alveolar macrophage function may act synergistically to predispose the host to *Pneumocystis* pneumonia. *Pneumocystis* interacts with a number of lung cell types and mediates lung injury through a variety of mechanisms. For instance, *Pneumocystis* exhibits tight interaction with alveolar epithelial cells, which appears to promote proliferation of the organisms. Further, the binding of *Pneumocystis* to lung epithelium also suppresses the proliferative potential of these cells, thus slowing repair of damaged alveolar surfaces. In addition to cellular interactions, *Pneumocystis* also directly or indirectly affects the content and activity of lower respiratory tract proteins (e.g., fibronectin and vitronectin) and surfactant. The complex host-pathogen interactions that occur during *Pneumocystis* pneumonia illustrate that host defense requires a fine balance between the generation of an effective inflammatory response to clear infection

and maintenance of the integrity of the alveolar gas-exchange surface. Better understanding of these intricate processes will be necessary to design more effective therapeutic strategies for the management of this serious infection.

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10

T Cells in Defense Against *Pneumocystis*

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I. Introduction

Many groups of immunosuppressed individuals are susceptible to the opportunistic pathogen *Pneumocystis jiroveci*. In most but not all of these hosts, the primary immune derangement is a deficiency in the number or function of T cells [1]. While there are clearly humoral immune deficiencies that predispose hosts to *P. jiroveci* pneumonia, such as severe combined immunodeficiency, the majority of patients diagnosed with this infection are infected with HIV or are iatrogenically immunosuppressed [2]. Despite the clinical importance of *P. jiroveci* pneumonia, however, major gaps persist in understanding the host immunity to this organism. A recent National Institutes of Health (NIH) consensus conference identified several such unresolved issues concerning the host immune response to *P. jiroveci*. One of these issues concerned identification of the essential mechanisms of immune response to *P. jiroveci* in HIV-infected hosts and determination of whether responses remain localized or occur systemically [2]. This uncertainty extends to understanding the roles of T cells in defense against *Pneumocystis*. Among extracellular organisms, study of *Pneumocystis* demonstrates that T cells perform unique roles in control of host defense and pulmonary inflammation. Once impairments in pulmonary host defenses are understood, novel strategies

to correct these defects may be developed for treatment and prophylaxis of *Pneumocystis* pneumonia [2,3].

This chapter focuses on the roles of T cells in defense, considering the roles of CD4+ T cells, CD8+ T cells, and $\gamma\delta$ T cells. It must be acknowledged, however, that host defense against this pathogen consists of redundant and overlapping pathways. Contributions to host defense against *Pneumocystis* depend on the interactions of T cells with alveolar macrophages and alveolar epithelial cells (discussed in Chap. 9), by production of cytokines (Chap. 11), and by activating B cells for antibody production (Chap. 12). These interactions are predominantly controlled by T cells; therefore a review of human and animal investigations focused on changes in number and function of T cells provides an overview of coordination of defense against *Pneumocystis*.

II. CD4+ T Cells

A. CD4+ T Cells in Humans

Numbers of CD4+ T Cells in Humans

It has now been appreciated for many years that depletion of CD4+ T cells is a hallmark of HIV infection, and progressive depletion of CD4+ T cells during HIV infection correlates with the risk of developing *P. jiroveci* pneumonia [4]. There is some evidence that HIV viral loads also predict risk of *P. jiroveci*, but viral loads are not used routinely to assess risk or need for prophylaxis [5]. Furthermore, it remains unclear whether viral load is an independent predictor of risk of *P. jiroveci* pneumonia or only tracks with progressive depletion of CD4+ T cells [6].

Whether the extent of CD4+ T-cell depletion correlates with the severity or outcome of *P. jiroveci* pneumonia remains controversial. The fact that peripheral CD4+ T-cell counts may not reflect the numbers of CD4+ T cells in the lung available for defense against *P. jiroveci* further complicates prediction of severity. In a recent series, for example, numbers peripheral CD4+ T cells did not predict outcome in AIDS patients with *P. jiroveci* pneumonia, but low numbers of CD4+ T cells in bronchoalveolar lavages did indicate poor prognoses [7].

Recent data suggest that decreased CD4+ T cells counts also predispose patients without HIV infection to *P. jiroveci* pneumonia, although this patient population has been much less well studied [8]. In patients receiving chemotherapy for malignancy or immunosuppressive therapy after transplantation, median CD4+ T-cell counts were lower in patients with *P. jiroveci* pneumonia than in those without it. Furthermore, 91% of the patients with *P. jiroveci* pneumonia had peripheral CD4+ T-cell counts below 300/ μ L at the time of diagnosis. While confirmatory data are needed, this study suggests that monitoring of CD4+

T-cell counts in iatrogenically immunosuppressed patients may be useful in predicting risk of *P. jiroveci* pneumonia.

Recent work suggests that progressive depletion of CD4+ T cells also signals risk of colonization with *P. jiroveci*. Previously, identification of *P. jiroveci* organisms in respiratory secretions was deemed indicative of active infection. With more sensitive detection techniques, specifically polymerase chain reaction, it is apparent that carriage or colonization with *P. jiroveci* organisms occurs. Small numbers of non-HIV-infected patients have been identified as being colonized by *P. jiroveci* by polymerase chain reaction but without clinical evidence of pneumonia [9]. Colonization with *P. jiroveci* in these individuals was associated with peripheral CD4+ T-cell counts below 400/ μ L and with CD4+/CD8+ ratios below 1. Thus, CD4+ depletion may predispose hosts to colonization prior to clinical pneumonia.

If depletion of CD4+ T cells is indeed the primary risk factor for development of *P. jiroveci* pneumonia, it is logical that immunological reconstitution should decrease the risk of pneumonia. Highly active antiretroviral therapy (HAART) has decreased the incidence of pulmonary infections in HIV-infected individuals dramatically [10], and successful immune reconstitution with HAART dramatically decreases the incidence of *P. jiroveci* pneumonia [11]. Discontinuation of primary prophylaxis [12,13] and secondary prophylaxis [14,15] appears to be safe for HIV-infected individuals with adequate responses to HAART. In fact, the most recent U.S. Public Health Service recommendations suggest that providers should discontinue primary and secondary *P. jiroveci* prophylaxis for sustained increases in CD4+ T-cell counts above 200/ μ L for at least 3 months [16]. Whether viral load (HIV RNA) should be considered as an independent trigger for prophylaxis is controversial [17], and additional longitudinal data are needed.

The frequency of *P. jiroveci* pneumonia in HIV-infected individuals has decreased, but there is some evidence that HAART influences the CD4+ T-cell counts at which individuals become susceptible to *P. jiroveci*. This risk is particularly apparent in those individuals who respond suboptimally to HAART. The Centers for Disease Control monitored risk of *P. jiroveci* pneumonia in patients receiving HAART as part of the Adult and Adolescent Spectrum of Disease Project [10]. During the 1992–1998 period, patients who developed *P. jiroveci* pneumonia while receiving HAART had higher median CD4+ T-cell counts than patients who had never received HAART. However, the magnitude of these differences was small (CD4+ T-cell counts of 29 vs. 13/ μ L, respectively), so their clinical importance is uncertain.

For those individuals who do respond to HAART with significant increases in numbers of CD4+ T cells, it is possible that immune reconstitution would change the clinical course of *P. jiroveci* pneumonia. An immune reconstitution inflammatory syndrome has been described in HIV-infected individuals with

other pulmonary infections. However, such manifestations appear to be uncommon for *P. jiroveci* pneumonia and the published literature is limited to case reports and small series. For example, a patient receiving HAART with a CD4 + T-cell count of 525/ μ L developed fever, malaise, and progressive dyspnea [18]. A lung biopsy demonstrated *P. jiroveci* pneumonia with an exuberant, granulomatous inflammatory reaction. An additional syndrome has been described in which patients are treated successfully for cytologically proven *P. jiroveci* pneumonia, HAART is initiated with successful immune reconstitution, and recurrent pneumonias occur without detection of any pathogen [19]. The authors speculate that reconstitution of immune responses, including increased numbers of CD4 + T cells, produce inflammatory reactions to residual *P. jiroveci* antigen in the lung. However, amounts of residual *P. jiroveci* antigen were not measured. It is also possible that small numbers of intact organisms remained that were below the threshold for cytological detection but could have been identified by polymerase chain reaction.

Taken together, these studies confirm that depletion of CD4 + T cells results in susceptibility to *P. jiroveci* pneumonia and that immunological reconstitution restores resistance to infection. Further work will be needed to determine whether immunological reconstitution changes the clinical features of pneumonia and whether restoration of inflammatory responses is beneficial or detrimental to the host.

Function of CD4+ T Cells in Humans

The mechanisms by which CD4 + T cells enable elimination of *P. jiroveci* by the host are poorly understood [20]. It is clear that exposure in early life to this ubiquitous organism results in antibody formation in the majority of humans [21], as discussed in Chapter 12. The development and duration of cell-mediated immunity in immunocompetent hosts is less well studied. T cells from immunocompetent adults recognize and proliferate in response to *Pneumocystis* antigen in vitro, but T cells from the cord blood of neonates do not respond [22]. These proliferative responses are antigen-specific and depend on the presence of monocytes as antigen-presenting cells. Therefore it is likely that cell-mediated immunity to *P. jiroveci* is acquired early in life, as is humoral immunity.

Presumably, exposure to *P. jiroveci* in early life results in the formation of memory cells and a diverse repertoire of clones that can proliferate when reexposed to *P. jiroveci*. The mechanism by which HIV infection depletes *P. jiroveci*-specific T-cell clones has recently been investigated. It is possible that HIV acts by vertical depletion, removing selected clones in the repertoire. Much more likely is the possibility that HIV acts by horizontal depletion, reducing the numbers of memory cells in each progeny without affecting the numbers of clones in the repertoire. Recent work suggests that the latter mechanism occurs [23].

Expansion of *P. jiroveci*-specific CD4+ T-cell clones in vitro demonstrated that clonal diversity is preserved, but overall numbers of antigen-specific cells are reduced during HIV infection.

The preceding data confirm that quantitative defects in CD4+ T cells directed against *P. jiroveci* predispose to infection, but are there also qualitative defects? Although experimental work has been directed toward answering this question, uncertainty persists. Peripheral blood mononuclear cells from HIV-infected individuals demonstrate decreased proliferation in response to *Pneumocystis* in comparison to cells from uninfected individuals [24]. Equal numbers of peripheral blood mononuclear cells from each individual were examined, but the investigators did not evaluate possible differences in numbers of CD4+ T cells in the preparations. The lack of response in cells from HIV-infected individuals was not specific to *Pneumocystis*, because decreased proliferation was also measured in response to tetanus toxoid and to phytohemagglutinin. Additional work demonstrated that proliferative responses of human peripheral blood mononuclear cells to *Pneumocystis* correlated directly with numbers of peripheral CD4+ T cells in HIV-infected individuals [25]. Therefore it remains unclear whether the lack of proliferative response was qualitative or dependent on decreased numbers of CD4+ T cells in the preparations.

Reduced numbers (and possibly reduced function) of CD4+ T cells during HIV infection result in susceptibility to *P. jiroveci* pneumonia, but *P. jiroveci* also modulates replication of HIV. Thus, *P. jiroveci* may induce enhanced viral proliferation, further decreasing the numbers of CD4+ T cells available for defense. For example, increased HIV-1 replication was detected in peripheral blood mononuclear cells during severe *P. jiroveci* pneumonia [26]. This enhanced viral replication was specific to *P. jiroveci* pneumonia, because no enhanced viral replication was observed in patient with herpes simplex virus or cytomegalovirus infections. Patients with *P. jiroveci* pneumonia have increased viral RNA in cell-free bronchoalveolar lavage (BAL) fluid compared with asymptomatic HIV-infected individuals [27]. Furthermore, the increase in viral replication was compartmentalized to the lung, as increases in viral RNA exceeded those in the sera of these patients. Additionally, HIV p24 antigen production was increased in alveolar lymphocytes from HIV-infected individuals with *P. jiroveci* pneumonia compared with lymphocytes from HIV-infected individuals without pneumonia [28].

Taken together, these data indicate that cell-mediated immunity directed against *P. jiroveci* occurs in humans and that decreased proliferative responses of CD4+ T cells directed against *P. jiroveci* occur during HIV infection. Additionally, *P. jiroveci* pneumonia increases HIV replication in the lung, which could further lower the numbers of CD4+ T cells available to participate in host defense. Clearly, further work is required to examine the qualitative capabilities of these responding CD4+ T cells.

B. CD4+ T Cells in Animal Models

Importance of CD4+ T Cells in Animal Models

As the previous section demonstrates, difficulties in studying human cells present formidable methodological obstacles to direct testing of hypotheses about CD4+ T cells and their function in vivo. Conversely, animal models permit modulation of T cells and their subsets in vivo, allowing direct testing of specific hypotheses concerning the role of T cells in defense against *Pneumocystis* [29]. Early studies of the host immune response to *Pneumocystis* were performed in rats [30] and other laboratory animals [31,32] chronically treated with corticosteroids to render them susceptible to infection. Work with these models showed associations between the depletion of T cells caused by corticosteroids and susceptibility to *Pneumocystis* pneumonia as well as improved defense against *Pneumocystis* when corticosteroid administration was discontinued [33–35]. Furthermore, these studies prepared the field for mouse models by documenting that *Pneumocystis* pneumonia in corticosteroid-immunosuppressed rats resulted in decreased percentages of T-helper cells and increased percentages of T-suppressor cells in the lung. However, the diverse effects of corticosteroids on immunity made it difficult to isolate the effects of individual immune effector mechanisms.

Animal studies have confirmed the importance of CD4+ T cells in host defense against *Pneumocystis*. Spontaneous outbreaks of *Pneumocystis* pneumonia occurred sporadically in colonies of athymic mice (which lack T cells) and SCID mice (which have severe combined immune deficiencies and lack T and B cells), indicating that T-cell deficiency rendered mice susceptible to *Pneumocystis* [36]. The use of monoclonal antibodies directed against CD4, which deplete CD4+ T cells in vivo, demonstrated that depletion of CD4+ T cells allows infection, while repopulation with CD4+ T cells cleared infection [37,38]. In these models, direct inoculation of mouse-derived *Pneumocystis* was used to induce infection, although CD4-depleted mice are also susceptible to environmental acquisition of infection. Depletion of CD4+ T cells can also be achieved by injecting the hybridoma cells directly into mice [39]. Conversely, inoculation of *Pneumocystis* into immunologically intact mice results in accumulation in the lung of both CD4+ and CD8+ T cells [40,41], followed by complete clearance of infection.

Expanding on the observation that SCID mice experience sporadic outbreaks of *Pneumocystis* pneumonia, these mice have also been used to investigate the roles of CD4+ T cells in defense against *Pneumocystis* [38–42,43]. Additionally, SCID mice can be immunologically reconstituted with T cells to determine the outcome of reconstitution on the course of infection. Reconstitution with CD4+ T cells clears infection [44], but depletion of CD4+ T cells from the reconstituting T cells abrogates this effect [38]. It appears that cellular reconstitu-

tion completely clears the infection without evidence of latency, even during subsequent depletion of CD4+ T cells [45].

Furthermore, CD4+ T-cell knockout mice are also susceptible to *Pneumocystis* [46–48]. Mice lacking conventional CD4+ T cells (H-2I-1 β knockout), T cell–receptor $\alpha\beta$ cells (TCR β knockout), and all peripheral T and B cells (RAG-1 knockout) are all exquisitely susceptible to *Pneumocystis*.

Despite the strict species specificity of *Pneumocystis* organisms, human CD4+ T cells can provide host defense against *Pneumocystis* in mice. Human CD4 transgenic mice (which express human CD4 but not murine CD4) are resistant to *Pneumocystis* when exposed to infected SCID mice [49]. If the transgenic mice receive a monoclonal antibody specific for human and chimpanzee CD4, the resultant CD4+ T-cell depletion renders the transgenic mice susceptible to murine *Pneumocystis* [49].

Even nonspecific immunosuppression may render mice susceptible to *Pneumocystis* pneumonia by depleting CD4+ T cells. For example, ethanol may have a permissive effect by inducing CD4+ T-cell dysfunction. Ethanol-fed mice developed *Pneumocystis* infections of low intensity, even in the presence of CD4+ T cells. The investigators speculated that ethanol decreased the number or function of CD4+ T cells [50]. Further work demonstrated that chronic ethanol administration decreases recruitment of CD4+ and CD8+ T cells to the lung and depleted numbers of splenic lymphocytes [51].

In summary, these animal investigations demonstrate that depletion of CD4+ T cells, or congenital absence of CD4+ T cells, renders mice susceptible to *Pneumocystis* pneumonia. Conversely, repopulation of endogenous CD4+ T cells or reconstitution with exogenous CD4+ T cells restores resistance to infection and clears previously established infection.

Function of CD4+ T Cells in Animal Models

It is clear that numbers of CD4+ T cells in experimental animals influence host susceptibility to *Pneumocystis* pneumonia, but the functional capabilities of these CD4+ T cells have not been defined completely. For purposes of organization, this section reviews current information regarding the *Pneumocystis* epitopes detected by CD4+ T cells, the role of antigen presentation, the effector functions of CD4+ T cells (including cytokine and chemokine production), production of lung inflammatory responses, and work in testing immunization strategies directed toward CD4+ T cells.

The specific *Pneumocystis* epitopes recognized by CD4+ T cells have not been characterized conclusively [52]. To determine whether T cells recognize and respond to *Pneumocystis* in a specific manner, most attention has focused on the major surface glycoprotein of *Pneumocystis* as a stimulating antigen. Lymphocytes from mice immunized with *Pneumocystis* proliferate in response to

purified major surface glycoprotein in vitro [53]. These specific proliferative responses occurred in cells from mice immunized with *Pneumocystis* or with purified major surface glycoprotein; they also occurred in cells from reconstituted SCID mice that had cleared the infection.

In rats, proliferation of splenic T cells occurs in response to major surface glycoprotein, but only in T cells obtained from rats previously exposed to *Pneumocystis* [54]. Further work demonstrated that these specific T-cell responses could also be elicited after immunization with a recombinant 55-kDa *Pneumocystis* antigen [55]. Adoptive transfer experiments have been used to extend these observations in vivo. Lymphocytes sensitized to the major surface glycoprotein confer resistance to *Pneumocystis* when transferred into corticosteroid-immunosuppressed rats [56]. However, transfer of major surface glycoprotein-specific CD4 + T cells produced a severe hyperinflammatory reaction with resultant mortality, as discussed below.

In order for CD4 + T cells to undergo clonal expansion in response to a pathogen, effective presentation of immunodominant epitopes by antigen-presenting cells must occur. Relatively little is known about the mechanisms of antigen presentation to CD4 + T cells during host defense against *Pneumocystis*. In general, antigen presentation to CD4 + T cells occurs in the context of appropriate major histocompatibility complex II (MHC II) expression, while antigen presentation to CD8 + T cells occurs in the context of appropriate MHC I expression. Mice lacking MHC II (H-2I-A β knockout mice), which have no conventional CD4 + T cells, are susceptible to *Pneumocystis* [46,57]. In contrast, mice lacking MHC I (β_2 -microglobulin knockout mice), which have no conventional CD8 + T cells, are resistant to environmental acquisition [46]. However, it is difficult to evaluate whether the absence of MHC molecules or the absence of the T cell subsets confers susceptibility or resistance.

Recent experiments demonstrate a role for antigen presentation in the lung during defense against *Pneumocystis*. Neonatal mice clear *Pneumocystis* more slowly and manifest less lung inflammation than adult mice. When reconstituted with splenocytes from adult mice, neonatal SCID mice clear *Pneumocystis* much more slowly than adult SCID mice [58]. One possible mechanism for this difference is that antigen presentation capabilities are not fully developed in neonatal lungs. Indeed, neonatal lungs contain fewer dendritic cells and Ia + (MHC II +) cells than adult lungs. When reconstituted with splenocytes from neonatal mice, neonatal and adult mice clear *Pneumocystis* equivalently [59]. These experiments suggest that the immunological competence of the lung environment (including antigen-presenting capabilities), rather than the competence of the transferred splenocytes, determines the success of host defense.

After CD4 + T cells have undergone clonal expansion, they must execute effector functions to become effective participants in host defense against *Pneumocystis*. As in other host responses, CD4 + T cells control a wide range of

cytokine and chemokine responses in the lung. For example, SCID mice with *Pneumocystis* pneumonia exhibit minimal lung inflammation histologically and do not mount significant cytokine or chemokine responses. When infected SCID mice are immunologically reconstituted with splenocytes, however, the inflammatory response results in increased lung mRNAs for IL-1 α , IL-1 β , IL-3, IL-6, interferon-gamma, TNF- α and TNF- β . Additionally, mRNAs for the chemokines lymphotactin, RANTES, MIP-1 α , MIP-1 β , MIP-2, and MCP-1 are induced [60]. These data demonstrate that T cells induce widespread inflammatory signals in response to *Pneumocystis*.

Whether CD4+ T cells induce cytokine responses that are predominantly Th1-like or Th2-like remains under investigation. Recent work suggests that a Th2 response predominates over a Th1 response [61]. Cytokine production by lung and lymph node CD4+ T cells after inoculation of immunologically intact mice with *Pneumocystis* was examined. In hilar and paratracheal lymph nodes, CD4+ T cells produced both interferon-gamma and IL-4 when examined 1 week after inoculation, and the response diminished over the next 3 weeks. In lung tissue, however, CD4+ T cells producing IL-4 predominated at early time points, followed by cells producing interferon-gamma. These data indicate that activation of CD4+ T cells occurs in lymph node prior to the appearance of these cells in the lung.

The isotypes of antibody responses to *Pneumocystis* have also been used to determine whether Th1 or Th2 responses predominate [62]. Mice were immunized with *Pneumocystis*, depleted of CD4+ T cells, and then rechallenged with *Pneumocystis* organisms. Despite CD4 depletion, the immunized mice cleared the infectious challenge. Wild-type mice inoculated with *Pneumocystis* mount IgG1 responses, with little production of IgG2a, IgG2b, or Ig3, indicative of a Th-2 type response. In contrast, IL-4 knockout mice mount primarily IgG2b responses, indicative of a Th1-type response. Because all groups of mice cleared the inoculum, the investigators argued that either Th1 or Th2 responses are sufficient for protection. Clearly more work is needed to understand the time course and mechanism of Th1 and Th2 CD4+ T-cell traffic in response to *Pneumocystis*.

In vitro, major surface glycoprotein stimulation has been used to examine the cytokine repertoire of CD4+ T cells obtained from rats environmentally exposed to *Pneumocystis*. By varying the time course and stimulating antigen, the investigators demonstrated that both Th1 (interferon-gamma) and Th2 (IL-4) cytokine production occurred [63]. Further cloning of the stimulated cells demonstrated that 5 clones produced Th1 cytokines (IL-2 and interferon-gamma), but one clone also produced small amounts of IL-4 [64].

The published literature establishes the importance of CD4+ T cells in decreasing infection, but the role of CD4+ T cells in control of lung inflammation during *Pneumocystis* is more controversial and may depend on the animal model [65]. In vivo, lymphocytes sensitized to the major surface glycoprotein confer

resistance to *Pneumocystis* when transferred into corticosteroid-immunosuppressed rats [56]. However, transfer of major surface glycoprotein-specific CD4+ T cells produced a severe hyperinflammatory reaction, with resultant mortality. In SCID mice, CD4+ T cells cause fatal hyperinflammatory reactions after reconstitution in some models [44]. Interestingly, this hyperinflammatory response is more pronounced after reconstitution using lymph node-derived cells from mice previously immunized against *Pneumocystis*. Histological localization of the CD4+ T cells showed that they aggregated in perivascular and peribronchial areas rather than in the parenchyma. This observation led the authors to speculate that the CD4+ T cells exert their detrimental effects by elaboration of soluble mediators rather than by direct damage at the alveolus. In later work, these investigators determined that administration of hyperimmune serum prior to the CD4+ T cells resulted in clearance of organisms but prevented the hyperinflammatory reaction [66]. It is tempting to speculate from this work that combined humoral and cellular approaches may be needed for successful immunotherapy of *Pneumocystis*.

Recent work may help explain why inflammatory responses to *Pneumocystis* have been beneficial in some systems but detrimental in others. Specifically, a better understanding of the phenotype and functional capabilities of the reconstituting cells may determine outcome. For example, a subset of CD4+ T cells that constitutively express the IL-2 receptor α chain (CD25) have been shown to prevent autoimmunity [65]. Transfer of CD25- cells into RAG-2 deficient mice infected with *Pneumocystis* cleared infection but caused lethal pulmonary inflammation. In contrast, transfer of CD25+ cells did not clear infection or produce fatal pneumonitis. Transfer of both CD25- and CD25+ cells cleared infection but prevented undesirable pulmonary inflammation. Thus manipulation of CD4+ T cell phenotypes to cause maximum organism clearance, while minimizing unwanted inflammation, may be feasible.

Finally, it is worthwhile to review a series of important investigations focused on immunization to increase CD4+ T-cell responses against *Pneumocystis*. Immunization of rats with recombinant *Pneumocystis* p55 antigen reduced organism burden in corticosteroid-immunosuppressed rats [67]. Although humoral responses were also elicited, splenocytes from the immunized mice proliferated in response to p55 antigen. The phenotype of the proliferating cells was not identified, but this work demonstrates that immunization can increase cellular as well as humoral responses. An immunization study examined the role of CD4+ T cells in host defense against *Pneumocystis* [68]. Intact mice were immunized with repeated intratracheal injections of *Pneumocystis* and then were depleted of CD4+ T cells. Rechallenge with *Pneumocystis* resulted in pneumonia in naive mice but not in the immunized mice. Immunization prior to T-cell depletion protected these mice against *Pneumocystis*, even when the mice were subsequently CD4-depleted.

In some models, immunological alterations can overcome persistent lack of CD4+ T cells. Additionally, administration of aerosolized, heat-treated *Escherichia coli* to CD4-depleted mice also results in clearance of infection [69]. In these experiments, lipopolysaccharide induced clearance of organisms that was dependent upon the presence of TNF- α . Although clearance did not depend on CD8+ T cells or natural killer (NK) cells, clearance depended on the accumulation of Thy-1+ CD4- CD8- cells in the lung. Whether these cells could be exploited for improved defense in CD4-depleted hosts is unknown.

Bone marrow-derived dendritic cells expressing CD40 ligand were pulsed with *Pneumocystis* antigen in vitro and then administered to CD4-depleted mice [70]. Despite the lack of CD4+ T cells, the mice receiving dendritic cells demonstrated significant titers of antibody against *Pneumocystis* and cleared the organisms from their lungs. Because transfer of serum from immunized mice into CD4-depleted, infected mice cleared infection, the investigators concluded that this immunization strategy works primarily by a humoral mechanism. Thus, it is possible that future immunization strategies directed toward humoral immunity can overcome cell-mediated deficits.

In summary, current understanding of the function of CD4+ T cells in animal models has progressed considerably, but major gaps persist. The immunodominant epitopes that elicit T-cell responses have not been characterized definitively. Recent work supports the likelihood that antigen presentation is important in defense against *Pneumocystis*, resulting in clonal proliferation of CD4+ T cells and differentiation into effector phenotypes. In some models, CD4+ T cells clearly have detrimental effects and cause fatal hyperinflammatory responses. Finally, immunization strategies directed toward enhancement of CD4+ T-cell function appear promising and need to be developed further.

III. CD8+ T Cells

A. CD8+ T Cells in Humans

Numbers and Function of CD8+ T Cells in Humans

As discussed above, the role of CD4+ T cells in defense against *Pneumocystis* in humans has been established, but the importance of CD8+ T cells in host defense against *Pneumocystis* has not been clearly defined. An infant with normal numbers of CD4+ T cells but absent CD8+ T cells who developed *Pneumocystis* pneumonia has been described [71], but such conditions are rare.

Increased numbers of CD8+ T cells are present in bronchoalveolar lavages obtained from many patients with AIDS; at least some of these T cells are cytotoxic cells directed against epitopes of HIV [72]. It is clear that CD8+ T cells in blood [73] and lung [74] can be infected with HIV and are likely to serve as

additional reservoirs for HIV infection. How these CD8 + T cells also participate in defense against *P. jiroveci* in humans is unknown.

B. CD8 + T Cells in Animal Models

Importance of CD8 + T Cells in Animal Models

In comparison to animal model data examining the role of CD4 + T cells in defense against *Pneumocystis*, the role of CD8 + T cells in decreasing the *Pneumocystis* burden is more controversial [38–44,75]. Inoculation of immunologically intact mice with *Pneumocystis* results in accumulation of both CD4 + and CD8 + T cells in the lung, followed by clearance of the organism [40,41]. In mice depleted of CD4 + T cells, inoculation with *Pneumocystis* results in massive accumulation of CD8 + T cells in the lung, although infection progresses [40]. Although large numbers of mice have not been examined, it does not appear that the accumulation of CD8 + T cells occurs in dexamethasone-treated mice with *Pneumocystis* pneumonia [76].

The CD8 + T-cell response to *Pneumocystis* pneumonia extends to primates, because SIV-infected macaques with *Pneumocystis* pneumonia demonstrate greater than 90% CD8 + T cells in broncholaveolar lavages during infection [77]. Because numbers of peripheral CD8 + T cells did not differ in SIV-infected macaques with or without *Pneumocystis* pneumonia, it is likely that the accumulation of CD8 + T cells in the lungs of these primates was in response to *Pneumocystis*.

Whether these lung CD8 + T cells have an important role in defense against *Pneumocystis* in animal models remains an area of controversy. Data from our laboratory demonstrate that CD8 + T cells contribute to defense against *Pneumocystis* during states of CD4 + T-cell depletion [75]. Mice were depleted of CD4 + T cells, CD8 + T cells, or both CD4 + and CD8 + T cells. Depletion of CD8 + T cells alone did not result in susceptibility to *Pneumocystis*. When both lymphocyte subsets were depleted, however, mice became more heavily infected with *Pneumocystis* than with depletion of CD4 + T cells alone. These data demonstrate that, in the absence of CD4 + T cells, CD8 + T cells provide partial defense against *Pneumocystis*.

A study using the corticosteroid-treated rat model strongly supports a role for CD8 + T cells in host defense against *Pneumocystis* [56]. Lewis rats, immunosuppressed with corticosteroids and infected with *Pneumocystis*, were reconstituted with CD8 + T cells obtained from the spleens of donor Lewis rats. When reconstitutions were performed with CD8 + T cells from donor rats exposed to *Pneumocystis* or stimulated in vitro with *Pneumocystis* major surface glycoprotein, the intensity of infection was reduced significantly. In contrast, if reconstitutions were performed with naive CD8 + T cells from unexposed rats, no differences in intensity of infection were observed.

In contrast, reconstitution experiments performed with SCID mice do not support an important role for CD8+ T cells in elimination of infection. As discussed above, SCID mice environmentally exposed to *Pneumocystis* clear organisms from their lungs when given splenocyte infusions [38]. In vivo depletion of CD4+ T cells from the splenocyte preparations abrogates this effect, but depletion of CD8+ T cells still permits clearance. In reconstitution experiments that utilized SCID mice infected with *Pneumocystis*, the mice did not clear infection when CD8+ T cells are administered, but they cleared infection when CD4+ T cells were administered [44]. Furthermore, mice genetically lacking CD8+ T cells (β_2 -microglobulin knockout) are not susceptible to *Pneumocystis* [46].

Therefore, whether modulation of CD8+ T-cell numbers alters susceptibility to *Pneumocystis* in animal models remains controversial. It is likely that differences in the method of CD8+ T-cell manipulation (depletion vs. reconstitution), method of acquisition of *Pneumocystis* (environmental vs. inoculation), timing of infection, and mouse strain differences all contribute to discrepancies in the published literature.

Function of CD8+ T Cells in Animal Models

In mice, both CD4+ and CD8+ T cells participate in the development of cell-mediated immunity against *Pneumocystis*, as evidenced by the development of delayed-type hypersensitivity [78]. Mice immunized subcutaneously with *Pneumocystis* and then challenged with foot pad injections of *Pneumocystis* antigen demonstrate specific hypersensitivity at the site of challenge. Transfer experiments demonstrate that both CD8+ and CD4+ T cells must be administered for delayed-type hypersensitivity; transfer of single subsets does not confer hypersensitivity.

The mechanisms by which CD8+ T cells function in control of *Pneumocystis* infection and inflammation in the lung are largely unknown. The lung CD8+ T cells that accumulate in the lungs of CD4-depleted mice infected with *Pneumocystis* proliferate in vitro in response to *Pneumocystis* antigen [75]. Because this proliferation depends on the presence of macrophages, it is likely that antigen-presenting cells are able to process and present antigen to lung CD8+ T cells. Additionally, these lung CD8+ T cells elaborate interferon-gamma in response to *Pneumocystis* antigen, pointing to a possible mechanism by which CD8+ T cells participate in host defense.

In fact, the host-defense capabilities of CD8+ T cells against *Pneumocystis* can be augmented by overexpression of interferon-gamma, resulting in clearance of infection even with prolonged depletion of CD4+ T cells [79]. In these studies, gene transfer was used to increase lung interferon-gamma expression on infected, CD4-depleted mice. This overexpression produced additional recruitment of

CD8+ T cells and NK cells to the lung and resulted in clearance of infection. Additionally, the presence of CD8+ T cells was required for clearance, because gene transfer into infected SCID mice or into mice depleted of both CD4+ and CD8+ T cells did not result in clearance.

More recently, these investigators examined the role of CD8+ T cells in mice deficient in $\gamma\delta$ T cells [80]. In comparison to wild-type controls, these $\gamma\delta$ T-cell deficient mice demonstrate increased clearance of *Pneumocystis*, increased recruitment of CD8+ T cells, and increased interferon-gamma production. Neutralization of interferon-gamma or depletion of CD8+ T cells prevented the increased clearance. These data, then, confirm the supplemental role of CD8+ T cells in host defense.

In contrast to these beneficial effects, several investigations implicate the CD8+ T cell in the induction of undesired and detrimental lung inflammation. CD8+ cells have been implicated in derangements of surfactant function that occur during *Pneumocystis* pneumonia. The experimental literature clearly demonstrates that *Pneumocystis* pneumonia induces surfactant dysfunction (reviewed in Chap. 13). Importantly, *Pneumocystis* reduces levels of the hydrophobic surfactant proteins B and C, leading to increased alveolar surface tension, atelectasis, and hypoxia [81]. Other investigators have found no alteration in surfactant biophysical activity in SCID mice infected with *Pneumocystis* [82]. When these infected mice are reconstituted with splenocytes, however, the inflammatory response decreases the biophysical activity of surfactant. Importantly, CD8+ T cells drive this response, because reconstitution with CD8-depleted splenocytes abrogates this dysfunction.

Additionally, the same investigators determined that CD8+ T cells, and the lung inflammation they produced, altered lung compliance and oxygenation [83]. Mice depleted of CD4+ T cells developed *Pneumocystis* pneumonia with intense inflammation, decreased oxygenation, and decreased lung compliance. In contrast, mice depleted of both CD4+ and CD8+ T cells developed pneumonia but did not develop physiological derangements. In this system, then, the CD8+ T cells recruited in response to *Pneumocystis* induced lung damage.

To summarize, much more work must be done to establish the functional roles of CD8+ T cells against *Pneumocystis* in animal models. The discrepancies in the published literature suggests that the roles are quite complex. It is likely that, under some circumstances, CD8+ T cells demonstrate beneficial effects and aid in clearance of organisms. In other circumstances, however, it is clear that CD8+ T cells induce detrimental lung inflammation.

IV. Gamma-Delta T Cells

A. Gamma-Delta T Cells in Humans

Most T cells, in the blood and in the lung, express $\alpha\beta$ T-cell receptors on their surfaces, including most CD4+ and CD8+ T cells. In both compartments, a

minority of T cells express $\gamma\delta$ T-cell receptors, usually without expression of CD4 or CD8 molecules. Numbers of peripheral blood $\gamma\delta$ T cells are reported to be decreased in HIV-infected individuals with *P. jiroveci* pneumonia or other opportunistic infections [84], while other investigators found that the percentages of circulating $\gamma\delta$ T cells are increased in patients with *P. jiroveci* pneumonia [85]. Conversely, the percentages of $\gamma\delta$ T cells in peripheral blood and in bronchoalveolar lavage are increased in HIV-infected individuals with *P. jiroveci* pneumonia [86]; in another study, percentages of $\gamma\delta$ T cells in the lungs of patients with *P. jiroveci* pneumonia were also increased [87].

B. Gamma-Delta T Cells in Animal Models

Mice lacking $\gamma\delta$ T cells (TCR δ knockout mice) are resistant to environmentally acquired *Pneumocystis* infection [46]. Interestingly, in these studies, large numbers of $\gamma\delta$ T cells appeared in the lungs of mice lacking $\alpha\beta$ T-cell receptors (TCR β knockouts). The investigators speculated that appearance of the $\gamma\delta$ cells represented an “immunological salvage” response to infection in an effort to compensate for lack of $\alpha\beta$ T cells. These authors went on to demonstrate that mice lacking both $\alpha\beta$ and $\gamma\delta$ T cells (TCR $\beta \times \delta$ knockouts) are exquisitely susceptible to *Pneumocystis* pneumonia. These mice still mounted inflammatory reactions, consisting of B cells and NK cells [48].

More recently, the role of $\gamma\delta$ T cells in responding to intratracheal inoculation with *Pneumocystis* has been examined using knockout mice [80]. In comparison to wild-type controls, these $\gamma\delta$ T cell-deficient mice demonstrate increased clearance of *Pneumocystis*. The increased clearance was accompanied by increased recruitment of CD8 $^{+}$ T cells and increased interferon-gamma production. Neutralization of interferon-gamma, or depletion of CD8 $^{+}$ T cells, prevented the increased clearance.

V. Summary and Conclusions

This chapter has reviewed the current literature examining the roles of CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, and $\gamma\delta$ T cells in host defense against *Pneumocystis*, both in humans and in experimental models. Overall, it is clear that depletion of CD4 $^{+}$ T cells renders hosts susceptible to *Pneumocystis* pneumonia and that repletion of CD4 $^{+}$ T cells restores host defense and resistance to infection. The actual mechanisms by which CD4 $^{+}$ T cells provide defense are poorly understood, and the immunodominant epitopes eliciting responses are not known. Nevertheless, immunization strategies directed toward modulation of CD4 $^{+}$ T-cell function may be useful in therapy or prevention of infection. It also seems clear that CD8 $^{+}$ T cells are not required for defense against *Pneumocystis* when CD4 $^{+}$ T cells are present in adequate numbers. In the absence of CD4 $^{+}$ T cells,

however, the role of CD8+ T cells is much more controversial. It seems likely that CD8+ T cells have beneficial effects, resulting in some clearance of organisms, but they also induce detrimental lung inflammation and damage. Finally, the role of $\gamma\delta$ T cells needs much more investigation, but recent work suggests that $\gamma\delta$ T cells participate effectively in defense against *Pneumocystis*. Further investigation of the roles of T cells in defense against this important opportunistic pathogen should lead to novel immunotherapies for the prevention and therapy of *Pneumocystis* pneumonia.

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11

Cytokines

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I. Introduction

Cytokines are polypeptide mediators with diverse and often overlapping biological activities. Released by all nucleated cells, cytokines provide a mechanism for intercellular communication that is not dependent on cell contact. Cytokines rarely function in isolation but participate in cascades or networks of cytokines released in response to an infectious challenge. This cascade of cytokine release results in variable concentrations of different cytokines at variable times within a particular tissue. The resultant cytokine milieu in the tissue regulates all aspects of the host response to infection. In the early innate immune response to infection, cytokines activate resident cells to phagocytize an infectious pathogen and regulate recruitment of additional inflammatory cells from the vasculature. During the evolution of the adaptive immune response, cytokines influence the type of lymphocyte response generated both locally and in regional lymphoid tissue. Cytokines also circulate to the bone marrow to stimulate a leukocyte response to infection. During infection, most of the activity of cytokines takes place within the infected tissue. However, with extensive infection or as a result of pathogen virulence factors, cytokines may be released from the tissue compartment and exert toxic effects on the host. Clinically, these systemic effects of cytokines may be manifest as

sepsis, shock, or the adult respiratory distress syndrome. Thus, cytokines are involved in every aspect of host defense against infection and play a determining role in whether an infection is cleared or results in death of the host.

Cytokines may be arbitrarily classified according to biological activity as follows:

- Proinflammatory
- Lymphocyte-derived
- Chemokines
- Colony-stimulating factors

This chapter summarizes current concepts about these classes of cytokines and pulmonary infection with *Pneumocystis*. As with all investigative work with *Pneumocystis*, much of our current knowledge is based upon animal models; relatively little information is available about cytokines in human *Pneumocystis jiroveci* infection.

Infection with *Pneumocystis* involves almost exclusively the alveolar space, where *Pneumocystis* organisms attach to alveolar epithelial cells. Tissue invasion or extrapulmonary spread is rare. With this in mind, cellular sources of cytokines during *Pneumocystis* infection are limited to cells within the alveoli, either resident cells or inflammatory cells recruited from the vascular compartment. In *Pneumocystis* pneumonia, the trophozoite form of *Pneumocystis* binds tightly to the surface of *alveolar epithelial cells*, mostly type 1 pneumocytes [1] and possibly type 2 pneumocytes [2]. Incubation of *Pneumocystis* with alveolar epithelial cells in vitro stimulates release of Interleukin-6 (IL-6) [3] and chemokines [4]. Other resident cells include *alveolar macrophages*, which provide a phagocytic barrier to inhaled pathogens and are potent sources of cytokines. Alveolar macrophages can internalize and kill *Pneumocystis* both in vivo [5] and in vitro [6,7]. Culture of *Pneumocystis* with alveolar macrophages in vitro stimulates release of the proinflammatory cytokines, tumor necrosis factor [8–11], IL-6, and IL-1 beta [12]. Infection with *Pneumocystis* is associated with early infiltration of lung tissue by *neutrophils*; in patients also infected with HIV, this correlates with a poor prognosis [13]. Neutrophils have the capacity to internalize and kill *Pneumocystis* in vitro [14], but the role of neutrophil-derived cytokines in the pathogenesis of *Pneumocystis* infection remains unknown. Because host defense against *Pneumocystis* is critically dependent on *Tlymphocytes*, it has long been accepted that the protective function provided by these cells is mediated in part through cytokine release. Cytokine release by T lymphocytes could take place in regional (hilar) lymph nodes or at the site of infection within the alveolar space.

II. Proinflammatory Cytokines

Proinflammatory cytokines can activate macrophages to increase their antimicrobial functions, augment antigen presentation, or induce chemokine expression,

which results in recruitment of inflammatory cells into lung tissue. Proinflammatory cytokines are discussed in two contexts: first, elaboration in response to the infection itself and, second, in the context of immune reconstitution in either human subjects or experimental animal models. Proinflammatory cytokines, which control the influx of inflammatory cells in the lung in response to *Pneumocystis* infection, include interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor (TNF).

IL-1 is released in response to many pulmonary infections and is implicated in the development of fever [15] and sepsis [16]. It is present in the bronchoalveolar lavage fluid of HIV-infected patients with *Pneumocystis jiroveci* pneumonia pneumonia compared to controls [17], whereas its antagonist, IL-1 receptor antagonist protein, is elevated in the serum of the same patients. Moreover, Chen and colleagues have reported that IL-1 activity increases rapidly in the lungs of reconstituted SCID mice with mouse-derived *Pneumocystis* infection, and that administration of an antibody, which prevents binding of IL-1 to its receptor, completely abrogates resolution of infection after spleen cell reconstitution [18]. However, IL-1 and other proinflammatory cytokine elaboration are insufficient by itself to mediate host resistance against natural *Pneumocystis* infection in the absence of $\alpha\beta$ + T cells or mature T and B cells. For example, Hanano and colleagues have investigated acquisition of *Pneumocystis* infection in a variety of mutant strains of mice with defective T-cell function, such as class II MHC knockout mice, or RAG-1 $-/-$ mice [19]. Despite production of TNF, IL-1, and nitric oxide (NO) in these models, these mice are highly permissive of natural infection by mouse-derived *Pneumocystis*, suggesting that a robust innate immune response in the absence of CD4 + T cells is insufficient to prevent *Pneumocystis* infection.

IL-6 is released by alveolar epithelial cells in response to *Pneumocystis* attachment, and data to date suggest that IL-6 can modulate the inflammatory response to infection. In the SCID mouse reconstitution model, IL-6, which can downregulate IL-1 and TNF, is present in the serum and lungs of infected mice, but administration of an antibody to IL-6 has no effect on the clearance of *Pneumocystis* infection [20]. Interestingly, infected mice treated with the IL-6 antibody have significantly more inflammatory cells in lung tissue, suggesting that IL-6 might downregulate inflammation associated with *Pneumocystis* infection in mice.

Among proinflammatory cytokines, much work has focused on TNF in *Pneumocystis* infection. TNF is a proinflammatory cytokine, produced in the lungs principally by alveolar macrophages but also by alveolar epithelial cells. *Pneumocystis* induces release of TNF from normal human monocytes [8] and alveolar macrophages in vitro [9,10] and from murine alveolar macrophages in vivo [21]. Release of TNF in response to an acute challenge with mouse-derived *Pneumocystis* peaks at 3 hr and precedes the influx of inflammatory cells into

the lung [21] (Fig. 1). Moreover, *Pneumocystis* induction of TNF appears to be independent of toll-like receptor 4 (TLR4) signaling, since TNF can be elicited by an acute challenge of *Pneumocystis* in C3H/HeJ mice. This strain of mice has a point mutation in the cytoplasmic domain, which prevents TLR4 signaling [22]. In fact, recent work by Vassallo and colleagues show that the β -glucan in the *Pneumocystis carinii* cyst wall can mediate the production of TNF in vivo [23] and by alveolar macrophages in vitro [24], presumably through the β -glucan receptor.

Data from both humans and experimental animal models suggest a protective role for TNF in *Pneumocystis* infection. Krishnan and colleagues have demonstrated that TNF is present in the bronchoalveolar lavage fluid of HIV-infected patients with *Pneumocystis jiroveci* pneumonia and that the TNF levels inversely correlate with cyst burden [25]. Pesanti has reported that TNF binds to *Pneumocystis carinii* [26] and is directly lethal to the organism in vitro [27]. Confirmatory data regarding this direct toxicity are lacking [28]. However, TNF is clearly required for adequate host defense against this pathogen in vivo. Chen and colleagues have demonstrated that the use of an anti-TNF antibody delays resolution of murine *Pneumocystis* infection in SCID mice reconstituted with spleen cells, suggesting that endogenous TNF is critical for resolving the infection [29]. Moreover, neutralization of TNF using a soluble TNF inhibitor results in delayed clearance of murine *Pneumocystis* in CD4+ T-cell replete mice and significant exacerbation of infection in CD4-depleted mice [30]. These studies have been confirmed in mice with a homozygous deletion of the two known TNF receptors

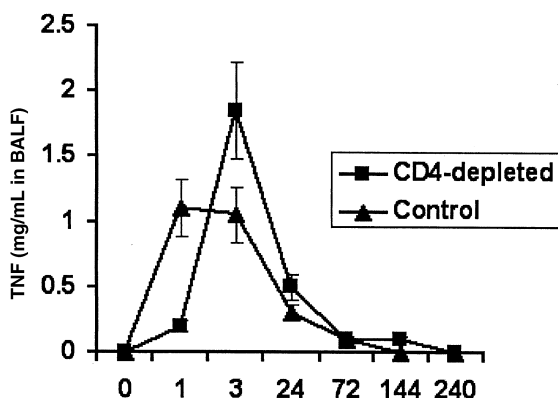


Figure 1 Elaboration of TNF in BAL fluid in response to murine *P. carinii*. 2×10^5 *P. carinii* cysts were instilled intratracheally in control or CD4-depleted mice. TNF was measured in BAL fluid at serial time points by ELISA ($n=4$ to 6 per group per time point). X axis is days after *P. carinii* instillation. (Adapted from Ref. 21.)

(TNFR I and TNFR II). These knockout mice can also clear an acute challenge with *Pneumocystis* [31]. However if these mice are crossed to IFN $-/-$ mice, which results in the loss of both TNF and IFN, these mice now become permissive for severe *Pneumocystis* infection[31], suggesting a potential coordinated role in host defense for these two cytokines. Moreover, treatment of CD4-depleted *Pneumocystis* infected mice with heat-treated *E. coli*, an inducer of TNF, causes near complete resolution of the infection[32]. Enhanced resolution of infection in this model could be abrogated with anti-TNF antibodies. Caution is warranted, however, regarding TNF as a potential immunotherapeutic agent. TNF is also temporally induced in *Pneumocystis*-infected lung tissue in the setting of immune reconstitution in mice and at high tissue concentration may cause lung injury in response to severe infection[33]. In fact, some investigators have postulated that pentamidine [10] or steroid-induced[34] decreases in TNF release by alveolar macrophages (AMs) may explain the improvement in *Pneumocystis jiroveci* pneumonia seen with these agents when used clinically.

III. Lymphocyte-Derived Cytokines

Lymphocytes and presumably lymphocyte-derived cytokines are closely linked to *Pneumocystis* infection; recruitment of lymphocytes to lung tissue is associated with clearance of infection while, conversely, deficiency in either the number or function of lymphocytes is associated with susceptibility to infection. In HIV-infected persons, the risk of PCP shows a linear and inverse correlation with the number of circulating CD4 $+$ lymphocytes [35]. The importance of the CD4 $+$ T lymphocytes in host defense against *Pneumocystis* is further supported by work with animal models. Experimental work from our laboratory shows that normal mice inoculated with *Pneumocystis* are able to resolve the infection without treatment, while mice depleted of CD4 $+$ T lymphocytes with a monoclonal antibody develop progressive infection [36]. When administration of the anti-CD4 antibody is stopped and CD4 $+$ lymphocytes are restored in lymphoid tissue, *Pneumocystis* organisms are cleared from lung tissue and infection resolves [37]. Collectively, this work along with the work of others [38,39] support a key role for the CD4 $+$ helper T lymphocyte in host defense against murine *Pneumocystis*. Yet CD4 $+$ lymphocytes are not the only lymphocyte active in host defense against *Pneumocystis*. In mice, *Pneumocystis* infection in CD4-deficient hosts is associated with markedly increased numbers of pulmonary CD8 $+$ lymphocytes [40]. The host defense role of these CD8 $+$ lymphocytes is not clear. Mice deficient only in CD8 $+$ lymphocytes are not permissive for *Pneumocystis* infection, but mice depleted of both CD4 $+$ and CD8 $+$ lymphocytes develop more severe infection [41]. These observations support a protective role for CD8 $+$ T lymphocytes that is only evident in the setting of CD4 $+$ T-lymphocyte deficiency.

All T lymphocytes do not have identical functional capacities, and the function of these cells is closely linked to the release of cytokines. Cloned lines of murine CD4 + T lymphocytes can be divided into functionally discrete subsets, designated Th1 and Th2, based on the types of cytokines produced *in vitro* [42,43]. Under this paradigm, Th1 cells produce IL-2, interferon gamma (IFN- γ), and tumor necrosis factor beta (TNF- β), while Th2 cells produce interleukins-4, -5, -6, 10, and 13 [44]. The cytokines IL- β , tumor necrosis factor alpha (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced by both subsets. CD8 + T lymphocytes may also be divided into similar functional subsets based on their cytokine profiles [45]. Th1 responses, through the elaboration of IFN and macrophage activation, favor cell-mediated immunity, while Th2 responses, through the elaboration of IL-4 and other cytokines, favor humoral immune responses [46,47]. Emerging evidence supports T-lymphocyte subsets in humans as well as mice [48,49].

The role of T-helper subsets in different disease processes is a subject of intense research, but it is clear that these subsets are important in host defense against infection. This has been best demonstrated in murine infection with *Leishmania major*, where mouse strains producing a Th1 response clear the infection while strains with a predominant Th2 response show disease progression [50,51]. Similar information has been reported in relationship to animal infection with *Mycobacterium tuberculosis* [52] and *Cryptococcus* [53] and to human infection with *Mycobacterium leprae* [54,55]. Collectively, these data support a protective role for a dominant Th1 lymphocyte response and a lesser role for a dominant Th2 response with regard to host resistance to infection.

There is limited information on T-lymphocyte subsets and *Pneumocystis* infection. Studies by Theus et al. demonstrated that *P. carinii* surface glycoprotein could stimulate splenic lymphocytes to release both IFN- γ (Th1) and IL-4 (Th2) *in vitro* [56]. Wright *et al* [57] found mRNA for IFN and IL-3 in lung tissue of infected SCID mice reconstituted with normal splenocytes. This is consistent with a Th1 response, although the cytokine mRNA was not localized to lymphocytes. This is an important limitation to interpretation, because nonlymphoid cells may also produce many lymphocyte-derived cytokines. On the other hand, work by Garvy et al. found that wild-type C57BL/6 mice challenged with mouse-derived *Pneumocystis* mounted an IgG1 antibody response, consistent with a Th2 response [58]. More direct evidence for T-lymphocyte subsets in murine infection comes from our laboratory [59]. In these experiments, normal BALB/c mice were inoculated with mouse-derived *Pneumocystis*. At serial intervals, CD4 + T-lymphocytes were purified from hilar lymph nodes and lung tissue and assayed for numbers of cells secreting IFN (Th1) and IL-4 (Th2) by ELISPOT assay. We found that the T-lymphocyte response to mouse-derived *Pneumocystis* is a mixed Th1 and Th2 response but is Th2-dominant in both regional lymph nodes and lung tissue. Although these results need confirmation in other mouse

strains and in humans, they suggest that host responses to *Pneumocystis* cannot be simply categorized as either Th1 or Th2.

Of the lymphocyte-derived cytokines, INF- γ has been most intensely studied in relation to *Pneumocystis* infection. Lymphocytes from normal rats exposed to *P. carinii* release INF- γ in vitro in response to *P. carinii* antigens [60] and glycoproteins [56], and increased numbers of CD4+ T lymphocytes releasing INF- γ can be demonstrated in both lymph nodes and lung tissue of normal mice challenged with mouse-derived *Pneumocystis* [59]. Even during states of CD4+ T-lymphocyte deficiency, CD8+ T-lymphocytes may protect the host from infection through elaboration of IFN. In CD4-depleted mice with progressive *Pneumocystis* infection, purified CD8+ T cells from lung tissue produce significant amounts of IFN in vitro, and IFN elaboration is further increased by stimulating the CD8+ T cells with *Pneumocystis* antigen [41]. Thus, *Pneumocystis* infection is associated with pulmonary CD4+ and CD8+ T lymphocytes with an enhanced capacity to release IFN.

In vitro studies show that IFN has biological potential to participate in host defense against *Pneumocystis*. For example, IFN enhances the ability of rat AMs to kill *P. carinii* [27] and increases TNF elaboration of rat AMs when the macrophages are exposed to rat-derived *Pneumocystis* organisms [10]. Thus, production of IFN may activate AMs for defense against *Pneumocystis* through augmented release of TNF. IFN may also activate other cells for defense against *Pneumocystis*. For example, human blood polymorphonuclear leukocytes demonstrate a respiratory burst when exposed to rat-derived *Pneumocystis*, and IFN enhances this response [61]. IFN also influences attachment of *P. carinii* organisms to alveolar epithelial cells, a process that may be important in pathogen clearance. IFN added to in vitro cultures inhibits attachment of rat-derived *Pneumocystis* to A549 cells, an alveolar epithelial cell line [62]. The mechanisms for this effect appears to be decreased expression of 5 and β 1 integrin subunits by the alveolar epithelium. Taken together, these in vitro data suggest that IFN could play an important role in defense against *Pneumocystis* by modulating host defense functions of macrophages, polymorphonuclear leukocytes, and alveolar epithelial cells.

Exactly how IFN participates in the pathogenesis of *Pneumocystis* infection in vivo has not been fully defined. Investigations in this area have utilized two experimental approaches: administration of IFN or the IFN gene to infected animals or neutralization of IFN activity in vivo using anti-IFN antibody or gene deletion (knockout). As an example of the first approach, BALB/c mice were specifically depleted of CD4+ T cells and infected with mouse-derived *Pneumocystis*. After *Pneumocystis* infection progressed for 4 weeks in these CD4-depleted mice, a subset of mice received recombinant IFN, administered daily as an aerosol for 2 weeks [63]. The lungs of the CD4-depleted mice treated with IFN demonstrated significantly less intense *Pneumocystis carinii* pneumonia (PcP) than the

lungs of CD4-depleted mice unexposed to IFN. In another animal model, corticosteroid-treated rats were environmentally infected with *Pneumocystis* and then treated with systemic IFN [64]. The intensity of PcP was significantly reduced in the IFN-treated rats and permitted reduced dosages of trimethoprim-sulfamethoxazole to clear the infection completely. These studies indicate that administration of exogenous IFN protein does not completely eradicate infection in immunocompromised hosts but does decrease organism burden. These results support but do not prove a similar role for endogenous IFN in host defense against this pathogen.

Administration of the IFN gene has been shown to upregulate host defense against *Pneumocystis* as well. In these experiments, CD4-depleted mice were administered the murine IFN gene in an adenoviral vector by intratracheal inoculation [65]. This results in high concentrations of IFN in lung tissue for up to 1 month after gene transfer. When CD4-depleted mice were treated with the IFN gene and then infected with mouse-derived *Pneumocystis*, it was found that the IFN gene transfer resulted in complete clearance of infection in spite of continued CD4 depletion. As expected, CD4-depleted mice administered a control gene developed heavy infection with *Pneumocystis*. The mechanism for this therapeutic effect of IFN appears to be mediated through a greater than fourfold increase in the number of pulmonary CD8+ T-lymphocytes in the mice receiving the IFN gene. Not only was the number of CD8+ lymphocytes increased, but their capacity to release cytokines was also upregulated, as reflected in increased IFN mRNA in purified CD8+ T lymphocytes from animals receiving IFN gene transfer. These results further support a host defense role for CD8+ T lymphocytes and suggest a new therapeutic approach for *Pneumocystic* pneumonia in immunosuppressed patients such as those with HIV infection.

On the other hand, experiments using antibody neutralization or gene deletion have generally failed to show a beneficial function for IFN during *Pneumocystis* infection. C.B-17 SCID mice environmentally exposed to mouse-derived *Pneumocystis* develop progressive pneumonia but clear the organisms when reconstituted with splenocytes. However, neutralization of IFN during reconstitution with monoclonal or polyclonal antibodies does not affect clearance of infection [29]. In other experiments, SCID mice were inoculated intratracheally with mouse-derived *Pneumocystis* and reconstituted with splenocytes obtained from normal or from IFN knockout mice [66]. Reconstituted mice resolved *Pneumocystis* infection whether splenocytes were obtained from intact or from knockout mice. Additionally, IFN knockout mice inoculated directly with mouse-derived *Pneumocystis* were able to clear an infectious challenge and did not develop pneumonia. Subtle differences in the rate of clearance between IFN knockout mice and control mice cannot be excluded. It should be noted, however, that mice lacking IFN demonstrated more intense pulmonary inflammation than control mice challenged with *Pneumocystis*. The mechanisms for this are not clear. Data

from these experimental approaches suggest that IFN is not essential for clearance of *Pneumocystis* organisms, but endogenous IFN may have a regulatory role in the tissue inflammatory response.

One explanation for the apparently inconsistent data on a host defense role for IFN may be that IFN does not operate alone in host defense against *Pneumocystis* but in concert with other cytokines. This concept is in keeping with what is known about cytokine networks during tissue infection [67]. Using knockout mice, Rudmann *et al.* showed such an interaction between IFN and TNF in host defense against murine *Pneumocystis* [31]. In these experiments, IFN-/- mice were able to clear infection, as were mice deleted of TNFR I and II (TNFR-/-). Thus, complete loss of signaling through either IFN or TNF was not sufficient by itself to preclude clearance of infection. However, simultaneous deletion of both genes (IFN-TNFR-/-) resulted in severe *Pneumocystis* pneumonia coupled with increased tissue inflammatory cells. These results support a host defense role for IFN against *Pneumocystis* in concert with other cytokines and reconcile apparently negative data from prior studies in the reconstitution SCID mouse model. They also serve as a reminder that inactivation of a single cytokine may not be deleterious if other cytokine pathways are operative to protect the host.

In summary, experimental findings to date support an important role for IFN in host defense against infection with *Pneumocystis*. Pulmonary host defense mechanisms are complex and redundant. IFN functions in vivo along with TNF and undoubtedly other cytokines to enhance host defense against this pathogen. Mechanisms through which IFN operates against *Pneumocystis* include but are not restricted to activation of phagocytic cells, decreased adhesion of *Pneumocystis* to alveolar epithelial cells, and enhanced recruitment of CD8+ T lymphocytes into lung tissue.

IV. Chemokines

Cytokines that stimulate leukocyte migration are termed *chemokines*. A major function of chemokines is to direct migration of leukocytes into infected tissues. The specific type of chemokine released in the tissue as well as the chemokine receptor expressed on the responding cell regulates the type of leukocyte that will enter a given tissue and also activates leukocytes at the site of chemokine release. More than 40 chemokines and 20 chemokine receptors have been identified [68,69]. Chemokine classification is based on the arrangement of cysteine residues in the amino-terminal domains [70]. CXC chemokines stimulate migration of neutrophils and T cells, while CC chemokines target monocytes, dendritic cells, eosinophils, and basophils. A variety of cell types have the capacity to release chemokines, but only limited information is available about cellular sources of chemokines in *Pneumocystis* infection.

Pneumocystis stimulates release of chemokines targeting migration of neutrophils *in vivo* and *in vitro*. In humans, the CXC chemokine IL-8 is a major chemoattractant for neutrophils. In HIV-related *Pneumocystis* pneumonia, IL-8 can be detected in lung lavage fluid [71], and the concentration of lavage fluid IL-8 correlates with more severe infection and increased mortality [72,73]. Furthermore, culture of *P. jiroveci* antigen with monocytes [74] and an alveolar epithelial cell line [4] *in vitro* results in release of IL-8. The mechanism through which *Pneumocystis* stimulates CXC chemokine release is mediated in part through interactions with beta glucan in the organism cell wall and alveolar macrophages. When purified beta glucan from rat *Pneumocystis* is cultured with alveolar macrophages, there is stimulated release of the CXC chemokine MIP-2 [23]. Chemokine release is blocked by incubation of the cells with alpha-mannan, suggesting that the beta glucan is acting through macrophage mannose receptors. Components of the *Pneumocystis* cell wall may also stimulate alveolar epithelial cells to release chemokines as well. When *Pneumocystis* surface glycoprotein is added to the A549 alveolar epithelial cell line, there is stimulated release of the CXC chemokine IL-8 and the CC chemokine MCP-1 [4]. Interestingly, glucocorticoids suppress this *in vitro* chemokine response, which may suggest a mechanism for the beneficial effects of glucocorticoid therapy in severe PcP [75]. Similar interactions between *Pneumocystis* cell wall components and lung cells may take place *in vivo* as well to recruit neutrophils into lung tissue. In support of this, direct instillation of beta glucan into rat lungs in the absence of intact microorganisms stimulates lung neutrophil influx and tissue inflammation [23].

Evidence for chemokine gene expression during *Pneumocystis* infection has also been demonstrated in the mouse system. Using a ribonuclease protection assay, Wright *et al.* assayed lung tissue from infected SCID mice for a battery of chemokines [76]. In heavily infected SCID mice, there was little chemokine mRNA in lung tissue. This is consistent with the inability of these mice to mount a tissue inflammatory response. However, when these infected mice were immunologically reconstituted with spleen cells from normal mice, mRNA for CC chemokines (RANTES, MCP-1, MIP-1 alpha, MIP-1 beta), for CXC chemokines (MIP-2), and a C chemokine (lymphotactin) were significantly elevated [76]. Presumably, this reconstitution model mimics the host defense mechanisms that permit clearance of infection in normal mice. Interestingly, mRNA for IP-10, which stimulates migration of Th1 lymphocytes, was not upregulated in the reconstituted mice, although mRNA for other chemokines with activity on Th1 cells was increased (MCP-1, MIP-1 alpha, MIP-1 beta, lymphotactin). These results need confirmation at the protein level, where the number of chemokine proteins translated during infection may be more limited than the number of chemokine genes expressed. However, it seems clear that local release of a variety of chemokines is a mechanism through which inflammatory cells are recruited to alveolar spaces

infected with *Pneumocystis*, and that chemokines are probably part of the normal host response to resolve infection.

V. Colony-Stimulating Factors

Colony-stimulating factors are a family of acidic glycoproteins required for the proliferation and differentiation of hematopoietic progenitor cells [77]. Three distinct recombinant human growth regulatory factors—granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF)—have been identified and cloned [78]. Both GM-CSF and G-CSF have been studied in the context of *Pneumocystis* infection.

GM-CSF, a cytokine that can activate mature macrophages and neutrophils, has been implicated in the host response to *Pneumocystis* pneumonia. GM-CSF is induced *in vivo* in response to an acute challenge with mouse-derived *Pneumocystis* [79], and GM-CSF can increase the respiratory burst (as measured by chemiluminescence) of neutrophils that have been exposed to *Pneumocystis* [61]. Paine and colleagues have demonstrated that GM-CSF is a critical regulator of the inflammatory response to *Pneumocystis* in murine lung. Mice with a homozygous deletion of the GM-CSF gene, which are subsequently CD4-depleted and challenged with *Pneumocystis*, develop intense inflammation compared to GM-CSF+/+ control mice. Furthermore local expression of GM-CSF in the lung, using a transgene drive by the lung specific surfactant protein C promoter, reduces the intensity of *Pneumocystis* infection as well the inflammatory response to the infection [79]. Data from our laboratory show that the systemic administration of GM-CSF protein reduces the intensity of *Pneumocystis* infection in CD4-depleted mice, perhaps through upregulation of TNF production in the lung [80] (Fig. 2). In conclusion, GM-CSF appears to be critical in regulating host defense against *Pneumocystis*, perhaps through enhanced TNF production, and thus may be a good candidate as an immunotherapeutic agent for adjuvant *Pneumocystis* therapy.

G-CSF specifically regulates the survival, proliferation, functional stimulation, and differentiation of precursor cells for granulocytes and also enhances the function of mature neutrophils [81]. G-CSF has recently been examined as a potential immunotherapeutic molecule in the steroid-induced rat model of *Pneumocystis carinii* infection. In this model, treatment with G-CSF afforded no protection against infection, whereas immunotherapy with CD40 ligand, a critical molecule in activation of antigen-presenting cells and in B-cell maturation, was effective [82]. Moreover, as excess neutrophils in the lung lavage fluid are a poor prognostic indicator in HIV-infected subjects with *Pneumocystis* pneumonia [83,84], G-CSF has the potential to exacerbate lung injury in this infection by increasing recruitment of neutrophils into infected lung tissue.

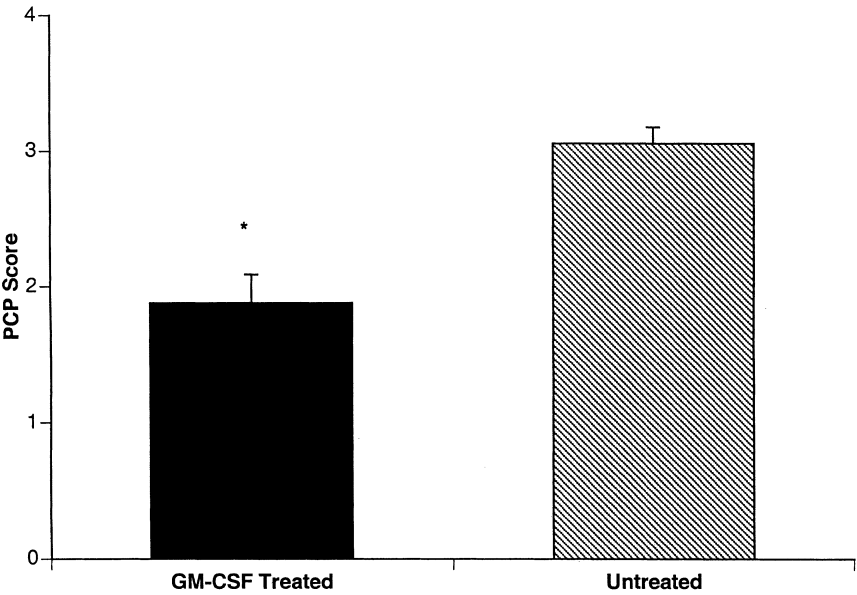


Figure 2 Intensity of *P. carinii* in CD4-depleted mice following 2 weeks of treatment with recombinant GM-CSF. $\pm$ SEM of 6 to 8 mice per treatment group. * = $p < 0.01$. (Adapted from Ref. 79.)

Table 1 Major Cytokines Implicated in Host Defense Against *Pneumocystis*

Class	Cytokine	References
Proinflammatory (innate immunity)	IL-1	17–18
	TNF- α	25–32
Lymphocyte-derived (adaptive immunity)	IFN- γ	27,31,59,63–65 (<i>Pro</i>) 29,66 (<i>Con</i>)
	IL-4	59
Chemokines	IL-18 (MIP-2)	4,71–74,76
	MCP-1	4,76
Colony-stimulating factors	GM-CSF	61,79,80 ☐

VI. Summary

In conclusion, cytokines from all four biologically active classes (proinflammatory, lymphocyte-derived, chemokine, and colony-stimulating factors) have been demonstrated to participate in the host response to an infectious challenge with *Pneumocystis*. A summary of the major cytokines implicated to date in host defense against this pathogen is shown in Table 1. It should be emphasized that only a handful of potentially important cytokines have been studied for *Pneumocystis*, so much remains to be learned. In addition, most of the data available focus on individual cytokines. The roles of cytokine-cytokine interactions, changes in cytokine concentrations over time, and downregulation of potentially harmful cytokines in lung tissue represent important and largely unexplored aspects of host defense against *Pneumocystis*.

Abbreviations

AM: alveolar macrophage
HIV: human Immunodeficiency virus
IFN: interferon-gamma
IL-1: interleukin-1
IL-4: interleukin-4
IL-6: interleukin-6
IL-8: interleukin-8
GM-CSF: granulocyte/macrophage colony-stimulating factor
G-CSF: granulocyte colony-stimulating factor
MIP-2: macrophage inflammatory protein-2
PcP: *Pneumocystis carinii* pneumonia
Th1: T-helper 1 cells
Th2: T-helper 2 cells
TLR: toll-like receptor
TNF: tumor necrosis factor alpha

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The Role of Humoral Immunity in the Defense Against *Pneumocystis*

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I. Introduction

The immune system comprises a complex organization of innate and adaptive host defenses involving multiple organs. These include organ systems with mucosal surfaces, such as the respiratory and alimentary tracts, that encounter the bulk of the microbes to which we are exposed. The circulatory and lymphatic systems are classically thought of as contributing cellular and humoral adaptive immune mechanisms against infection. Nontraditional “immune” organ systems such as the central nervous and endocrine systems also participate in immunity against infection. As our understanding of immune defense mechanisms deepens, we come to see that the defense against most microorganisms is usually multifactorial. Thus, while it may be somewhat artificial to focus on a single aspect of the immune response in the defense against an infectious agent, such an approach may be useful in helping us to understand how various elements of host immunity can bring about control of an infection. In this chapter, we summarize the evidence that antibody-mediated immunity is critical in protecting against the development of *Pneumocystis* pneumonia (PcP), knowing full well that many components of the immune system interact to control disease produced by *Pneumocystis*. We also briefly describe examples of antibody-mediated immunity against other fungi as models that may provide a guide for future studies on *Pneumocystis*.

II. Observations in Humans

A. Seroprevalence and Age-Dependent Seroconversion to *Pneumocystis*

Serological screens of normal populations can often be an important tool to determine the prevalence of an infection in the population. Furthermore, seroconversion is usually a marker for resistance to subsequent infection provided that the individual's immunological mechanisms remain intact.

Cross-sectional studies have demonstrated that a significant portion of healthy individuals have antibody to *Pneumocystis* [1–3]. The pattern of antibody reactivity with antigens of *Pneumocystis* is related to the type of prior exposure [2,4]. Seroprevalence increases with the age of the population sampled. A longitudinal study, that followed a cohort of approximately 100 newborns in Santiago, Chile, demonstrated that only 16% of infants were seropositive at 2 months of age [5]. However, 50% of the cohort had seroconverted by 8 months of age; at final follow-up at 20 months of age, 85% of the children were seropositive (Fig. 1) [5]. This study was unable to associate typical respiratory illnesses with time of seroconversion or presence of *Pneumocystis* DNA in nasal secretions, most likely due to the large number of respiratory illnesses that occur during this period of life.

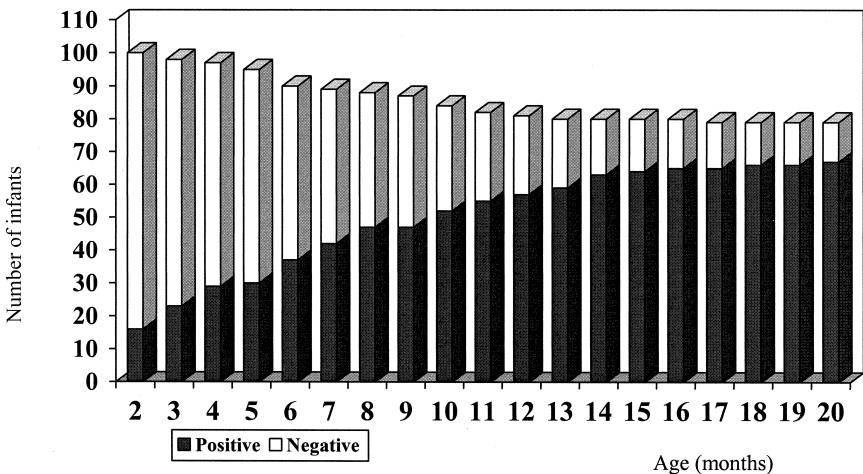


Figure 1 Proportions of infants with positive and negative ELISA titers of antibody to *Pneumocystis* at different ages in a cohort of 107 initially enrolled healthy infants. Of 100 infants tested at 2 months of age, 16 (16%) were seropositive, probably because of maternally acquired antibodies. Forty-seven (53%) of 88 infants and 67 (85%) of 79 infants continuing in the follow-up had seroconverted by 8 and 20 months of age, respectively.

In those infectious diseases where antibody is clearly an important determinant of immunity, seroconversion, either from active or subclinical infection, is a marker of protection against subsequent infection. In fact, this also seems to be the case with *Pneumocystis*. The majority of individuals are seropositive, and overt infections with *Pneumocystis* are virtually unheard of unless the host becomes immunocompromised.

B. Naturally Occurring Disease in Humans

Although serological data can be used to draw inferences about the role of antibody in protecting against infection with *Pneumocystis*, it does not provide any conclusive evidence that humoral immunity is a critical determinant of protection against PcP. However, observations of disease states in humans can provide more convincing evidence that disruption of humoral immunity is associated with a predilection for developing PcP. A review of reported cases of PcP in the pre-AIDS era demonstrated that patients with agammaglobulinemia were known to be at risk of developing PcP [6]. A common feature of the other immunodeficiencies reported in this review article is that they were generally associated with disturbances of antibody production, the most common being severe combined immunodeficiency (SCID) syndrome. Two subsequent reports of 6 children with hypogammaglobulinemia and PcP were important because the children in these reports did have some analysis of T-cell number and function performed, which did not demonstrate any gross abnormalities [7,8]. While these reports provide strong evidence that specific antibody is needed for protection against PcP, they do not rule out unrecognized defects in T-cell function. For example, the hyper-IgM syndrome, which has a phenotype of hypogammaglobulinemia, is a well-recognized immunodeficiency associated with PcP [9,10]. However, we now know that the molecular defect in this syndrome is a mutation in T-cell costimulatory pathways that interferes with normal T- and B-cell interaction during antigen presentation [9,10]. Thus, to gain definitive data regarding the role of the humoral immune system in protecting against PcP, it is necessary to turn to animal models, where experimental manipulation of various aspects of the immune response can be more carefully controlled.

III. Observations from Animal Models of *Pneumocystis* Pneumonia

One of the first observations of the antibody response to *Pneumocystis* in animal models of infection was reported by Walzer and Rutledge [11]. They showed that as corticosteroids were tapered after onset of PcP in corticosteroid immunosuppressed rats, there was a rise in serum antibody titers to *Pneumocystis* which correlated with a drop in organism burden. Furthermore, normal rats housed in

a common area with other *Pneumocystis*-infected rats developed a rise in serum antibody titers to *Pneumocystis* without evidence of active disease. More recently, we have performed time-course experiments in normal mice exposed to *Pneumocystis*-infected mice. These normal mice do develop an asymptomatic, pneumonitis, but with microscopically detectable organisms, by about 3 to 4 weeks after exposure that resolves spontaneously with onset of detectable serum antibodies to *Pneumocystis* (Fig. 2). Similar results have been obtained when normal mice are directly inoculated with *Pneumocystis*. In this model, organism numbers peak at 2 weeks postinoculation and then disappear by about 4 weeks, at which time serum antibody titer to *Pneumocystis* has peaked [12]. Together, these observations raise the question of whether the serum antibody rise after exposure to *Pneumocystis* mediates protection from overt disease or is merely a marker of exposure and clearance of *Pneumocystis* by other immune mechanisms.

More direct evidence for the necessity of B cells and antibody in protection against PcP comes from several mouse models of susceptibility to *Pneumocystis*. In SCID mice with PcP reconstitution with CD4 cells in the absence of B cells does not result in resolution of PcP, as it does when the reconstitution is done with a mixture of B and T cells. In these experiments the mice were shown to be able to reject skin grafts, demonstrating that they were T cell-competent [13].

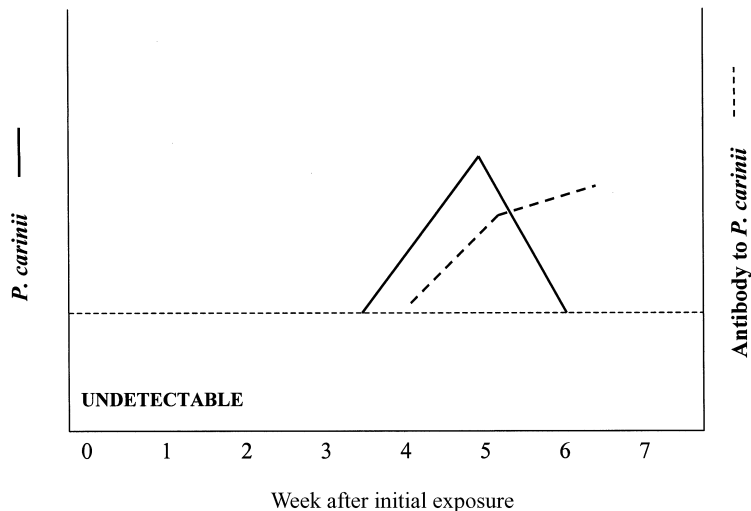


Figure 2 Kinetics of infection of normal mice by *Pneumocystis*. By approximately 3 weeks after commencing exposure to *Pneumocystis*-infected mice, organisms are microscopically visible in the lungs of the mice. Organism numbers peak at about 5 weeks and are not detectable by about 6 weeks. A measurable antibody response was elicited by 4 weeks after exposure, and it continued to rise as organisms disappeared.

Unambiguous evidence of the need for functional B cells to protect against PcP comes from studies with transgenic B cell-deficient muMT mice [14]. These mice have a disruption in one of the exons encoding IgM. This results in the inability to produce IgM—as well as IgG, A, and E—since these isotypes are derived by somatic rearrangement of the IgM gene. There is also failure of B cells to mature normally in these mice. Homozygous muMT(−)/muMT(−) mice develop fatal PcP if not maintained in a protected environment. In contrast, heterozygous muMT(+)/muMT(−) mice, which have normal immunoglobulins, do not develop PcP.

IV. Animal Models of Passive Immunization Against *Pneumocystis* Pneumonia

The most direct way to determine the role of a particular component of the immune response is to give back that element to animals unable to mount an active or acquired immune response to a given pathogen. Such an approach has been used in a variety of models of PcP to demonstrate that humoral immunity is an effective means to control PcP.

Before summarizing the animal model data demonstrating protection against PcP by antibody, it is of interest to review a human experience with passive immunoprophylaxis in an immunocompromised population. In a large trial of hyperimmune anti-cytomegalovirus (CMV) immunoglobulin to prevent CMV in renal transplant recipients, it was noted that in addition to having less CMV disease, the immunoglobulin recipients had a statistically significant drop in invasive fungal infections, including PcP [15]. While the study was not large enough to provide conclusive data with regard to passive immunoprophylaxis with antibody to prevent PcP, it did indicate that this is an area worthy of further investigation.

The initial demonstration that specific antibody can provide protection against *Pneumocystis* was done in corticosteroid-immunosuppressed rats and ferrets with PcP [16]. In this study, administration of a monoclonal antibody that binds the immunodominant *Pneumocystis* surface molecule glycoprotein A (gpA, also called the major surface glycoprotein or gp120) produced a 50 to 90% drop in organism numbers. Given that steroids suppress a variety of immune functions, the fact that passive administration of a single species of monoclonal antibody can reduce organism numbers demonstrates that humoral immunity likely has a role in the control of infection by *Pneumocystis*.

The importance of antibody was further demonstrated in studies of passive immunoprophylaxis using immunization-derived hyperimmune antisera to *Pneumocystis* [17,18]. In these studies, mice were made susceptible to *Pneumocystis* by depletion of CD4⁺ T cells. However, those animals administered polyclonal hyperimmune sera were substantially protected from infection. Of considerable

interest were the comparisons made between passive administration of hyperimmune antisera and *Pneumocystis*-sensitized normal lymphocytes [17]. In contrast to the high degree of protection afforded by the hyperimmune antisera, transfer of sensitized lymphocytes resulted in a high acute fatality rate, which was thought to be due to a hyperinflammatory reaction in the lung. Interestingly, pretreating mice with hyperimmune antisera before infusion of *Pneumocystis*-sensitized lymphocytes allowed for complete resolution of PcP without evidence of hyperinflammatory injury. The significance of this immune-mediated inflammatory lung injury is discussed in detail in Chapter 14 on mechanisms of lung injury during PcP.

We have recently experimented with the concept of topical application of monoclonal antibody to the respiratory tract as a means to protect against PcP [19]. Since PcP is an infection at a mucosal site acquired by inhalation of organisms, we hypothesized that it might be possible to use locally applied antibody to prevent disease. For these experiments, *Pneumocystis*-free SCID mice were cohoused with *Pneumocystis*-infected SCID mice for 2 weeks, resulting in transmission of infection to the *Pneumocystis*-free SCID mice. The *Pneumocystis*-free recipient mice received daily intranasal application of either *Pneumocystis*-specific monoclonal or control antibody in a 50- μ L volume. This volume would not only coat the nasopharynx but would also enter the lungs as the mice aspirated some of the applied material. Intranasal applications were continued for the 2-week period of cohousing plus an additional 3 days after the “seed” mice with PcP were removed. When sacrificed 6 to 7 weeks later, the mice receiving *Pneumocystis*-specific monoclonal antibodies had up to a 99% reduction in organism numbers, thus demonstrating the protective capacity of antibody in mice without functional B and T cells.

A potential limitation of antibody-mediated protection is antigenic variation by the organism. Organisms that can undergo antigenic variation are usually effective pathogens capable of evading the host immune response. Because *Pneumocystis* expresses a highly variable, immunodominant surface glycoprotein gpA, experiments were performed to see whether antigenic variation would occur in *Pneumocystis* in response to specific antibody exposure [20]. For these experiments, mice with PcP were given biweekly injections of monoclonal antibody 2B5, which binds to gpA. After 6 to 8 weeks, organisms were harvested from the mice for analysis and passage into new SCID mice, which were again treated with either monoclonal antibody 2B5 or irrelevant monoclonal antibody. Organisms were passed in this manner a total of three times for a total duration of exposure to monoclonal antibody 2B5 of 194 days. At each passage point, organisms were examined for the presence of the epitope recognized by monoclonal antibody 2B5 and compared to the epitopes recognized by other gpA-specific monoclonal antibodies. As the standard to identify *Pneumocystis* organisms in the homogenates and for comparison with reactivity to Mab 2B5, *Pneumocystis*

Mab 5E12 was used. Mab 5E12 is specific for a highly conserved carbohydrate epitope on gpA and reacts with virtually all organisms in a preparation.

As shown in Figure 3 [20], at each time point, approximately 60 to 75% of the Mab 5E12–positive organisms from mice treated with control antibody were also positive for the 2B5 antigen. In contrast, by day 91 (first forward passage), the percentage of 2B5 epitope-positive organisms dropped to 30% on organisms from those mice exposed to monoclonal antibody 2B5 *in vivo* and remained suppressed for the duration of antibody exposure. Interestingly, when antibody administration was stopped in one group of mice, expression of the 2B5 epitope began to increase toward the level that seen in control mice. In our earlier

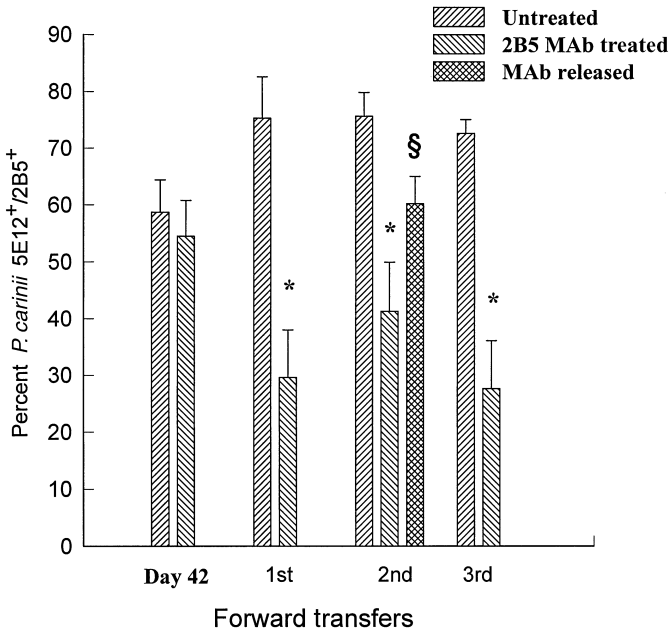


Figure 3 Percentage of epitope 5E12-positive *Pneumocystis* that were also epitope 2B5 fluorescence–positive by flow cytometry after three transfers into SCID mice. *Pneumocystis* organisms isolated from the lungs of SCID mice, which were either untreated or injected with 2B5 biweekly, were transferred into uninfected SCID mice three separate times, and the MAb pressure was either resumed or released (second transfer). Initial data were collected 42 days after initiation of the MAb pressure. Data from the first, second, and third forward transfers were collected at days 91, 147, and 194, respectively, after initiation of administration of MAb 2B5. Data represent the mean $\pm$ standard deviation ($n = 4$ to 5); *, $p < 0.05$ compared with untreated mice; §, $p < 0.05$ compared with 2B5-treated mice.

studies of passive immunoprophylaxis with monoclonal antibody 5E12, we noted a clear-cut reduction in cyst number. In this study, cyst numbers were not affected by giving monoclonal antibody 2B5, but trophozoite numbers were decreased 90% or more at each time point.

While *Pneumocystis* did demonstrate variation in gpA expression in response to specific monoclonal antibody administration, it was not classical antigenic variation, such as seen in the African trypanosome *Trypanosome brucei*. During *T. brucei* infection, organisms expressing a homogenous surface glycoprotein coat elicit an antibody response, resulting in destruction of the organisms. Those organisms that have undergone genetic rearrangement to express a new variable surface glycoprotein isoform escape attack, because the new surface glycoprotein is no longer recognized by the initial antibody response and multiply freely until they, in turn, elicit an immune response. For this type of antigenic variation to be effective, the organism must show a uniform antigenic profile to the host so that mutants can “escape” an immune response against the predominant population [21]. Since clonal populations of *Pneumocystis* cannot be derived, it is difficult to explain how gpA expression is varied. However, the results described above are consistent with the hypothesis that *Pneumocystis* organisms in a given population “randomly” express multiple, antigenically diverse gpA isoforms, and organisms are continually destroyed as they are recognized by the immune system. A host with intact immunity can effectively control infection by *Pneumocystis* by responding appropriately to multiple gpA isoforms. As immune competence wanes in the immunodeficient host, the ability to respond to different gpA variants in the population is slowly lost, allowing organisms to escape immune surveillance and colonize the lung.

V. Animal Studies of Active Immunization Against *Pneumocystis* Pneumonia

Studies of active immunization against PcP in animal models have also been published. Active immunization generally involves a combined B- and T-cell response. However, some of the studies were performed in such a manner as to demonstrate the role of humoral immunity in protecting against PcP. In one series of experiments, normal mice were immunized with whole *Pneumocystis* [22]. After an antibody response was documented, the immunized mice (and control mice) were depleted of CD4⁺ T cells and challenged with viable *Pneumocystis*. To ensure that the mice were effectively depleted of T cells, the mice were given antibody to both CD4 and Thy 1.2 beginning 10 days after immunization and continuing for 15 days prior to challenge. Immunized mice treated in this way were completely resistant to infection. Given that the mice were T cell-depleted and that cytokines would not be expected to remain elevated 25 days after the

last immunization, the presence of *Pneumocystis*-specific antibody would seem to be the most likely effector of the observed protection.

A follow-up series of experiments was done to examine the specificity of this protective antibody response for the immunizing strain of *Pneumocystis* [23]. In these experiments, if mice were immunized with ferret- instead of mouse-derived *Pneumocystis*, they were not protected when subsequently T cell-depleted and challenged with mouse-derived *Pneumocystis*. The failure of ferret-derived *Pneumocystis* to protect against a challenge with mouse-derived organisms was explained by the fact that immunization with ferret-derived *Pneumocystis* did not produce a measurable antibody response to mouse-derived *Pneumocystis*, despite developing the expected antibody response to ferret-derived *Pneumocystis*. This biological distinction between *Pneumocystis* derived from different mammalian hosts is consistent with the host species-specific phenotypic and genotypic variation among *Pneumocystis* discussed elsewhere in this book.

Intranasal active immunization against PcP has also been described [24]. Intranasal immunization with a crude preparation of soluble *Pneumocystis* antigens with the adjuvant cholera toxin fraction B resulted in protection when the immunized mice were subsequently CD4⁺ T cell-depleted and challenged with *Pneumocystis*. The protected mice mounted a strong antibody response to an antigen(s) with a molecular weight of 55 to 60 kDa. Finally, studies of immunization of interferon gamma or IL-4 knockout mice showed that an antibody response typical of either a Th1 or Th2 helper response was able to protect mice against PcP [25].

The common feature of all of these studies of active immunization is that mice were immunized in the presence of fully functional CD4⁺ T cells. Therefore one cannot dismiss the role that the T cells played in offering appropriate “help” for antibody production; however, antibody was likely the final effector of protection, since protection was demonstrated in the absence of T cells.

The most convincing demonstration that antibody-mediated protection can occur in the absence of CD4⁺ T cells comes from studies using dendritic cells transfected with CD40 ligand to “present” *Pneumocystis* to B cells [26]. For these studies, mouse bone marrow-derived dendritic cells expressing CD40 ligand, intended to trigger appropriate costimulatory signal in B cells, were pulsed *ex vivo* with *Pneumocystis* and then reinfused into CD4⁺ T cell-depleted mice. Despite being depleted of CD4⁺ T cells, the recipient mice developed a measurable *Pneumocystis*-specific antibody response. Most importantly, when these “vaccinated” mice were challenged with *Pneumocystis*, they demonstrated significant protection against infection. The fact that a protective antibody response could be produced without involvement of CD4⁺ T cells is strong evidence that the need for CD4⁺ T cells in maintaining resistance to PcP is to offer help in antibody production by B cells.

VI. Humoral Immunity to Other Fungi

The concept that humoral immunity is a significant factor in protection against PcP is relatively novel but not unique among fungal pathogens. Because *Pneumocystis* does not lend itself to in vitro studies or other types of specific manipulations, it is impossible to provide definitive data about the role of antibody in host-parasite interactions. Therefore studies of antibody-mediated protection against other fungi may provide useful analogies and insights for similar approaches to control the progression of PcP. The two fungi in which antibody-mediated protection has been studied in detail are *Candida albicans* and *Cryptococcus neoformans*.

The fungus *C. albicans* is a frequent commensal of the epithelial surfaces of the body, with the alimentary canal considered as the primary reservoir of colonization. No single virulence factor accounts for the transition of *C. albicans* from commensal to pathogen. A variety of different potential virulence attributes are likely to play a role at each stage of infection. While these potential virulence attributes may give *C. albicans* a competitive advantage, it is the host's immune competence that determines whether the fungus goes on to cause infection and disease.

An accumulating body of evidence has supported a role for specific antibody in protection against fungal infections, including *C. albicans* [27]. *C. albicans*-specific antibodies may provide protection against the first phases of epithelial colonization by either agglutinating organisms, leading to clearance, or by blocking fungal adhesins [28]. Once epithelium is breached by penetration of fungal elements, these defense mechanisms would be expected to quickly lose their efficacy. However, since the fungus has penetrated the epithelium, cellular and humoral immune components may interact to control the infection. Antibodies may function either directly or by fixation of complement as an opsonin for professional phagocytes [27], such as polymorphonuclear leukocytes (PMNs) [29] and macrophages [30], cells that are known to exert a protective effector function against *C. albicans*.

Both protein and carbohydrate moieties of the cell wall are capable of eliciting a strong humoral response, which may include antibodies that provide protection [31]. Hence, a better understanding of antibody reactivity with cell wall antigens of *C. albicans* may provide the basis for therapy against infection. In support of this concept, passive immunization with *C. albicans*-specific antibody has been successful in reducing the level of colonization and infection in a number of cases. Han et al. have used a monoclonal antibody to a mannan cell wall component that both agglutinated the organism and inhibited adhesion to host macrophages [32,33] to protect mice against disseminated candidiasis and vaginal candidiasis. In the mouse model of disseminated candidiasis, passive immunization with the protective monoclonal antibodies more efficiently bound

complement to the blastoconidium (yeast) form of *C. albicans* than did a nonprotective monoclonal antibody [34]. Antibody binding to the *C. albicans* surface results in activation of the classical complement pathway, followed by rapid deposition of the opsonin C3b [35,36], which further facilitates phagocyte binding to *C. albicans* and stimulates antimicrobial activity by the phagocyte. It was concluded that the protective potential of antibodies is dependent on epitope specificity, serum titer, and ability to efficiently fix complement to the fungal surface.

Phagocytes, in particular the PMNs, are critical for an effective response to disseminated fungal infection. Neutropenia and PMN dysfunction are critical to risk factors for the development of invasive fungal infection, including candidiasis [37]. Antibodies directed against the fungal surface have been demonstrated to enhance the function of PMNs. The same monoclonal antibody that protected mice against disseminated candidiasis also enhanced mouse PMN candidacidal activity [29]. Similar observations were seen with a monoclonal antibody specific for the capsular polysaccharide of *C. neoformans* [38]. Further, bispecific antibodies directed against the *C. albicans* surface, and either IgG (CD64) or IgA (CD89) receptors on human PMNs, enhanced the killing of the fungus by PMNs [37]. In support of a role of opsonization in killing of *C. albicans* by PMNs is the observation that reduction in IgG receptors led to decreased candidacidal activity [38].

Tavares et al. [41] also obtained protection against drug-resistant strains of *C. albicans* using monoclonal antibodies in a mouse model of disseminated infection. Cassone and colleagues have demonstrated passive protection against vaginal infection in mice with vaginal fluid containing antibodies to mannan and the aspartyl proteinase of *C. albicans* [42]. Tollemar et al. reduced fungal colonization in bone marrow transplant patients by oral administration of bovine anti-*Candida* antibodies [43]. Not all antibodies directed to the *C. albicans* surface have been protective [30]. However, the number of recent examples where specific antibodies have mediated protection suggest that a better understanding of the specificity and characteristics of protective antibodies against *C. albicans* may lead to new strategies for controlling candidiasis.

C. neoformans causes disease primarily in immunocompromised patients. *C. neoformans* can be either an intracellular or extracellular pathogen. In individuals with normal immune defenses, *C. neoformans* is usually found in macrophages and giant cells, and the method for controlling the infection is granulomatous inflammation. In the immunocompromised host, *C. neoformans* is frequently found in extracellular spaces and the infection does not stimulate a strong inflammatory response [44]. Like *Pneumocystis*, immune protection against *C. neoformans* was classically thought to be due mainly to cell-mediated responses. Attempts to confer protection against infection using polyclonal sera gave conflicting results; hence the role of antibodies in protection was considered minor

at best. However, in the past two decades, a role for antibody-mediated protection against *C. neoformans* has emerged from studies using monoclonal antibodies and a mouse model of infection (reviewed in Ref. 27).

Passive administration of an IgG1 monoclonal antibody prolonged survival and reduced tissue burden in mice infected with a lethal dose of organisms [45,46]. A primary target of protective monoclonal antibodies against *C. neoformans* is the anti-phagocytic polysaccharide capsule of the fungus, which is composed largely of glucuronoxylomannan (GXM). Using monoclonal antibodies, protection against infection is strongly influenced by antibody isotype. The relative efficacy of IgG subclasses in protection against *C. neoformans* is IgG2a/IgG1 > IgG2B >> IgG3 [47,48]. IgG3 monoclonal antibodies to *C. neoformans* are nonprotective and in some circumstances can actually enhance disease [49]. A nonprotective IgG3 directed against the GXM capsule was converted to a protective antibody by isotype switching to an IgG1 [50].

Epitope specificity is also an important determinant of protection against *C. neoformans*. Studies on two IgM monoclonal antibodies derived from a single B cell demonstrated that one IgM was protective while the other was not. The two IgM antibodies differed in several amino acid residues in their respective antigen binding sites as a result of somatic mutation introduced during the development of the antibody response [51].

An added level of complexity to monoclonal antibody-mediated protection against *C. neoformans* infection in the mouse has been revealed by recent studies. The IgG1 monoclonal antibody that protects against *C. neoformans* challenge in normal mice loses its protective capacity in SCID mice and in CD4⁺ T cell-deficient mice [52]. Interestingly, the nonproductive/disease-enhancing IgG3 monoclonal antibody is protective in CD8⁺ T cell-deficient mice. The results seen in both the CD4⁺ and CD8⁺ T cell-deficient mice can be reversed by administration of interferon gamma [32]. A more detailed analysis of the role of specific cytokines in the modulation of antibody efficacy against *C. neoformans* [53] demonstrated that both Th1- and Th2-associated cytokines were involved in antibody-mediated protection.

Therefore, in a mouse model of infection, the efficacy of certain monoclonal antibodies in protection against *C. neoformans* is dependent on antibody isotype, epitope specificity, and T-cell function. Furthermore, the participation of T cells in protection is markedly influenced by cytokines. Together, these studies reveal a degree of interaction between the humoral and cellular arms of the immune system that has been little appreciated in the context of protection against *C. neoformans* infection.

VII. Summary

Pneumocystis is a true opportunistic pathogen that produces overt disease only in the compromised host. Defects in CD4⁺ T-cell number or function are a

common predisposing factor for the development of PcP. However, these defects are associated with abnormal antibody responses as well. Whether or not antibody is the primary means of protection against PcP in the normal host, animal models have clearly established that specific antibody can provide protection against PcP.

It should be determined whether T cells and Th-specific cytokines also modulate the outcome of infection following administration of monoclonal antibodies in models of candidiasis and PcP. Furthermore, the participation of complement in antibody-mediated protection against PcP remains to be adequately investigated. The dynamics of the interplay between antibodies, complement, T cells, and phagocytes profoundly influences the efficacy of the immune response against fungal infections in general. Understanding how to manipulate the outcome of this interplay in favor of the host, particularly in those whose body defenses are compromised, will be a significant challenge for researchers designing immunotherapeutic strategies against pathogenic fungi.

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The Pulmonary Surfactant System in *Pneumocystis* Pneumonia

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I. Introduction

A major cause morbidity and mortality in patients suffering from *Pneumocystis* pneumonia (PcP) is the associated impaired gas exchange and resultant clinically significant hypoxemia [1]. The chest radiograph in PcP is often underwhelming in comparison to the alveolar-arterial oxygen gradient, suggesting the presence of severe intrapulmonary shunting and microatelectasis. In addition, altered lung mechanics and increased work of breathing are present.

Nearly 50 years ago, the pioneering work of both Pattle and Clements each provided compelling evidence relating the pressure-volume properties of the lung to the surface tension–surface area behavior of the alveolar lining material, leading to the convincing argument for the presence of a pulmonary “surfactant” (meaning *surface active agent*) [2,3]. Soon thereafter, Avery and Mead published the first clinical description of altered surface tension in association with prematurity of newborn infants [4]. Since that time we have become increasingly appreciative of the multiplicity of clinical syndromes associated with dysfunction of the surfactant system [5–7].

Pulmonary surfactant is a surface-active, biochemically heterogeneous mixture of phospholipid and protein secreted by the lung epithelium that reduces surface tension at the air-liquid interface and allows for maintenance of alveolar

stability at low lung volumes [8]. Based on a voluminous literature on the composition, structure, and function of lung surfactant that has accumulated since its discovery in pulmonary edema fluid, lung slices, and broncho alveolar lavage (BAL) fluid, the diversity of structure, function, and metabolism of individual surfactant components has been elucidated. Early research initiatives focused primarily on describing the composition, biophysical properties, and metabolism of the phospholipids (reviewed in Refs. 9 to 11). Much of the protein fraction in isolated surfactant was found to consist of serum proteins; however, a small but important group of lung-specific proteins have been described [12]. On the basis of function, these surfactant proteins can be divided into two groups: (1) SP-B and SP-C are small lipophilic proteins that have been shown to be involved in augmentation of adsorption of phospholipids at the air-liquid interface, in monolayer organization, and in intracellular lipid packaging [13], while (2) larger, more hydrophilic proteins, SP-A and SP-D, are multimeric glycoproteins that do not appear to have a primary role in the reduction of surface tension. In the past decade, however, these proteins have become recognized as important members of a growing family of host defense molecules, the collagen-like lectins ('collectins'), which participate in innate lung immunity through interactions with a variety of organisms, including *Pneumocystis* [14,15].

Although the pathophysiology of hypoxemic respiratory failure in PcP remains incompletely understood, studies both in humans and in animal models of infection suggest that abnormalities in hydrophobic component expression and in the biophysical activity of pulmonary surfactant play an important role in the altered respiratory physiology associated with this infection [5,16,17]. Furthermore, many recent investigations localizing the expression of SP-A and SP-D to small airways and alveoli of the distal respiratory tract [18–22], the demonstration of binding of these proteins to *Pneumocystis* [23–26], and evidence showing that both SP-A and SP-D can interact with and modulate the function of macrophages and lymphocytes [27–29] make these proteins ideal candidates as local lung host defense molecules against *Pneumocystis* lung infection.

This chapter examines the increasingly complex and important role of lung surfactant in both the biophysical function of the lung and the host defense/pathogen response to the *Pneumocystis* organism. The basic biochemistry and cell biology of the surfactant system is reviewed, the effect of PcP and the contribution of the associated host inflammatory response to surfactant component expression and function are described, and the emerging concept of the role of surfactant in both the innate and adaptive response to *Pneumocystis* lung infection is discussed.

II. The Surfactant System: Components and Functions

The study of surfactant component expression during PcP depends upon fractionation of the surfactant material for accurate quantification. As shown in Figure 1,

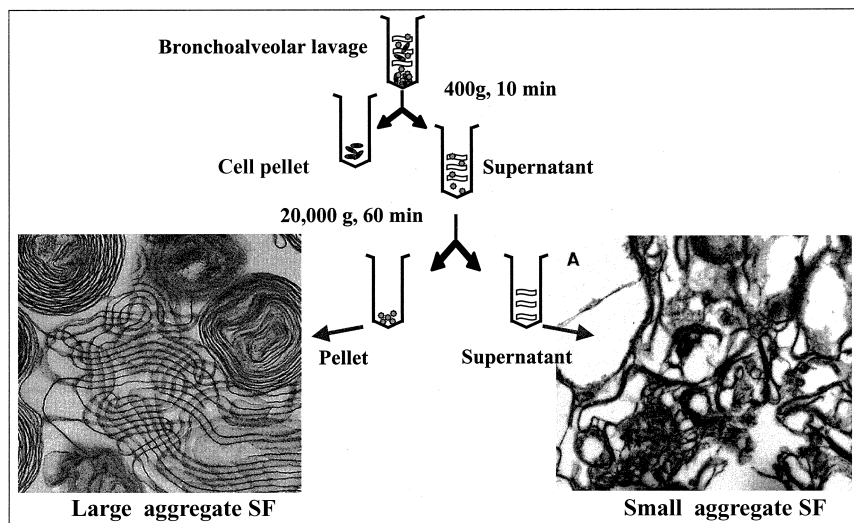


Figure 1 Isolation of surfactant fractions from bronchoalveolar lavage. BAL containing cells and protein is subjected to low-speed centrifugation to isolate a cell pellet. The cell-free supernatant is further fractionated via high-speed centrifugation into the biophysically active large-aggregate (LA) form and small-aggregate (SA) fraction. Representative electron micrographs of surfactant aggregate forms show the presence of tubular myelin and multilamellated vesicles in the LA while the small aggregate fraction contains unilamellar vesicles.

using differential centrifugation, a cell-free bronchoalveolar lavage obtained by low-speed centrifugation can be further subfractionated into “large-aggregate” (LA) and “small-aggregate (SA) pools [30–34]. A number of groups have shown morphological and functional differences between large and small surfactant aggregates. Tubular myelin, large multilamellar vesicles, and lamellated bodies are observed in the LA fraction, which has also been shown to account for the biophysical properties of surfactant. In addition, a majority of the SP-A and all of the SP-B and SP-C are found in this fraction. In contrast, SA fractions contain only small unilamellar vesicles and show poor surface activity both *in vitro* and *in vivo*. The majority of SP-D and some SP-A have been found in this subfraction [35].

The reason for this distinction of surfactant subtypes extends beyond morphological or biochemical characteristics. Several groups have noted a functional correlation between surfactant aggregate subtypes and the development of lung dysfunction. The “conversion” of surfactant subfractions (i.e., a decrease in the percentage of phospholipids in LA and an increase in SA) has been shown to

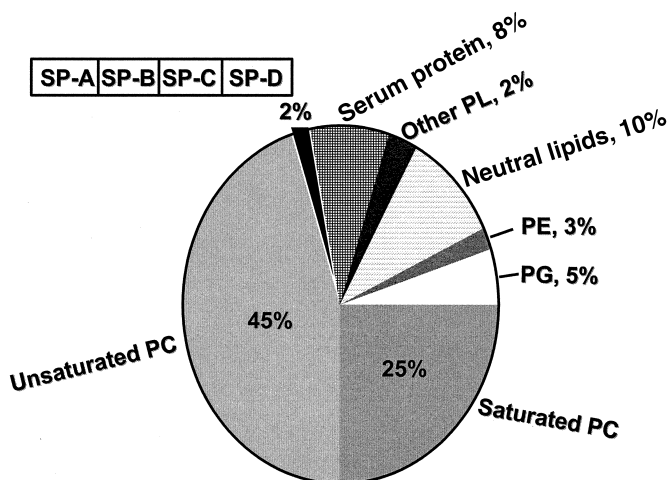


Figure 2 Composition of pulmonary surfactant. Approximate composition of large-aggregate surfactant is shown as weight percent. These values are approximate; exact composition can vary with species of donor, method of isolation, and immune status.

occur in several models of experimental lung injury and in human patients with the acute adult respiratory distress syndrome (ARDS) [27,31,32,36,37] as well as in PcP.

Despite these more recent investigations highlighting distinctions in BAL surfactant subfractions, it has been the LA form that has traditionally been studied as “surfactant.” This cell-free, lipid-rich pellet obtained following high-speed centrifugation has been analyzed from a variety of species and on average, by weight, consists of 90% lipids and 10% proteins [12]. The major subcomponents are schematically summarized in Figure 2.

A. Phospholipid

Surfactant lipid is predominantly enriched in disaturated phosphatidylcholine (DPPC), monounsaturated or lysophosphatiolylcholine (lyso PC), as well as smaller amounts of phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) (reviewed in Ref. 11). Minor amounts of other lipid classes (including cholesterol and sphingomyelins) can be also recovered. Surfactant phospholipids are produced by the alveolar type II cell through a combination of *de novo* synthesis utilizing choline as well through recycling and reutilization of surfactant lipids from the alveolar space via regulated reuptake (reviewed in Ref. 38). Assembled phospholipid generated from both *de novo* synthesis and recycling/reutilization is stored in subcellular organelles (lamellar

bodies) of type II cells prior to release into the alveolus. Phospholipid secretion from type II cells occurs via a regulated exocytosis and can be stimulated by a variety of agents and second messengers acting through both protein kinase C (phorbol esters, ATP) and protein kinase A pathways (terbutaline, cAMP) as well as via increases in intracellular calcium [10,39]. Many of these same compounds are known to stimulate surfactant synthesis as well.

Type II pneumocytes are also a major pathway for clearance of surfactant lipid. By a variety of techniques, labeled DPPC or its analogues has been shown to enter alveolar epithelial cells and accumulate in lamellar body fractions [38,40,41]. As for secretion, uptake can be modulated by a variety of secretagogues, including terbutaline, cAMP, and TPA, suggesting that the two processes are linked to help facilitate homeostasis in surfactant pool sizes [9]. In addition to type II cells, alveolar macrophages have also been shown to participate in surfactant removal, being responsible for as much as 50% of the clearance of the lipid portion [42,43].

The bulk of the biophysical activity in lung surfactant—that is, the modulation of surface tension at the air-liquid interface of the alveoli and small airways—can be attributed in large part to the properties of the phospholipids. The biophysical activity of surfactant mixtures is assessed *in vitro* using a variety of techniques, including the Wilhelmy balance, pulsating bubble technique, or captive bubble surfactometer [44–46]. By these techniques, isolated surfactant is capable of developing rapid surface adsorption, achieving very high surface pressures, and developing low surface tension with the kinetics required to support *in vivo* observations of alveolar physiology and lung mechanics. However, it has been well recognized that mixtures of synthetic phospholipids that mimic the composition of natural surfactant are generally incapable of achieving this to the same degree and with the same kinetics as their natural counterparts. Thus, it was postulated and subsequently demonstrated that there are additional nonlipid components of pulmonary surfactant capable of augmenting the surface tension properties required of the phospholipid.

B. Surfactant Proteins

In 1973, Clements' group reported the isolation of a unique glycoprotein from the surface-active material obtained from canine lung lavage (variably termed SP26-35 or SAP-A)[47]. After this report, other smaller and more hydrophobic surfactant-associated proteins were soon described. In 1979, Phizackerly isolated a family of proteins from organic extracts of surfactant fractions of pig lung lavage and lung homogenate [48]. In 1984, A.B. Fisher's laboratory described a small hydrophobic peptide obtained from an ethanol-diethylether extraction of purified rat surfactant that their group termed Apo-Et [49]. Subsequent to these reports, other similar hydrophobic peptides (Molecular weight 3.5 to 8 kDa, re-

duced) were isolated from human, bovine, porcine, and canine sources [50–53]. A consensus conference in 1988 unified the nomenclature in which three proteins—the hydrophilic glycoprotein SP-A and the hydrophobic, biophysically active SP-B and SP-C—were recognized as surfactant-associated proteins [12]. Subsequent to the recommendations of this conference, a fourth protein localized to airway epithelia but associated predominantly with the high-speed supernatant of the BAL (SA surfactant) was isolated and termed surfactant protein D [54,55].

The monomeric form of each surfactant protein is shown in Figure 3. On the basis of structure, function, and solubility in organic solvents, surfactant proteins can be divided into two groups [12]. The primary and secondary structures of the two classes of protein (hydrophobic and hydrophilic) are markedly different. Additional comparisons of structure, posttranslational modifications, and functional properties of each protein are summarized in Table 1.

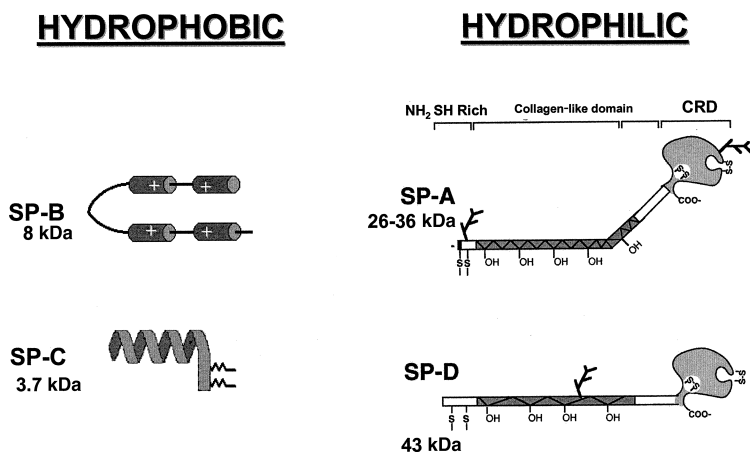


Figure 3 Monomeric structures of surfactant proteins. Schematic representations of monomeric forms of four surfactant associated proteins. At **left**, the hydrophobic proteins are depicted. SP-B is a member of the saposin-like family of peptides and contains seven cysteine residues, six of which promote intrachain disulfide bond formation, which induce a hairpin loop. SP-C is an apolar molecule with a membrane-spanning alpha helix that promotes intrinsic membrane association in lipid bilayers with an axis near perpendicular to the lipid acyl chains. At **right**, structures for two collection monomers are shown. Both proteins contain three regions each of similar size: an amino (NH₂) terminal region, a neck region, and a carbohydrate recognition domain (CRD). Differences in monomer size result primarily from differences in the length of the collagen-like domain that is longer in SP-D. An Asn-linked oligosaccharide is located in the collagen domain of SP-D whereas the Asn-linked sugar in SP-A is found within the CRD.

Table 1 Comparison of Structure and Function of Surfactant Proteins

Property	Hydrophobic			Hydrophilic	
	SP-C	SP-B		SP-A	SP-D
Structure					
M _r reduced (nonreduced), 10 ³	3.7 (6–7)	9 (18)		36 (72–700)	43 (130)
Primary translation product, kDa	21	42		26	39
Homology	None	Prosaposin		C1q ^b ; C lectins ^a ; collagen ⁺	collagen ^a ; C lectins ^a
Alpha helix	Yes	No		No	No
Membrane integration	Yes	No		No	No
Posttranslational processing					
Signal peptide	No	Yes		Yes	No
Propeptide cleavage (no.)	Yes (4)	Yes (3)		No	No
Glycosylation	No	Protein only		Yes (N-linked)	Yes (K-linked)
Palmitoylation	Yes	No		No	No
Functions^b					
Phospholipid adsorption	Yes (<SP-B)	Yes (>SP-C)		± (Needs SP-B)	No
Tubular myelin	No	Yes		Yes	No
Resistance to inactivation	Yes (<SP-B, >SP-A)	Yes		Yes	No
Monolayer formation	Yes (>SP-B)	Yes		No	No
Vesicle fusion	Yes	Yes		No	No
Host defense	No	+/- (proprotein)		Yes	Yes
Regulation of lipid secretion	No	No		Yes (↑)	No
Regulation of lipid uptake	±	±		Yes (↓)	Yes (in vivo)

^a Structural homology only.
^b Biophysical functions reviewed in more detail in Ref. 11.

Structure, Function, and Biosynthesis of Hydrophobic Surfactant Proteins

SP-B and SP-C are extremely lipophilic peptides shown to be involved in intracellular surfactant phospholipid packaging, extracellular surfactant monolayer organization, and augmentation of phospholipid absorption at the air-liquid interface. The contributions of SP-B and SP-C to physiological lung function and to the biophysical activity of surfactant lipid are underscored by several observations. First, the function of surfactant phospholipid monolayers at air-liquid interfaces cannot be explained by their surface properties alone (i.e., lipid adsorption, spreading, etc.) but rather require the presence of either natural SP-B or SP-C or one of several synthetic peptide analogues [56–58]. Second, intratracheal instillation of SP-B antibodies in rabbits creates respiratory distress syndrome (RDS) *in vivo* and inactivates replacement surfactants that contain SP-B [59–62]. Third, congenital deficiency of SP-B (and commensurate absence of SP-C) uniformly results in chronic respiratory failure in term human infants [63–67]. Fourth, respiratory failure in association with absent levels of SP-C and decreased levels of SP-B has been reported in the BWB breed of Belgian cows [68]. Fifth, in preterm animal models of RDS or in surfactant-depletion models of lung injury, replacement therapy using synthetic surfactants containing either SP-B or SP-C as the sole protein component produces biophysical properties similar to those of natural surfactants [69–72].

Surfactant Protein B

The cellular metabolism of SP-B has been substantially elucidated (reviewed in Ref. 13). The full-length SP-B mRNA (2.2 kb), produced by splicing of multiple exons, yields a predicted primary translation product 381 amino acids in length [73–75]. SP-B translation products of M_r 41,000 to 42,000 result from *in vitro* translation of human lung RNA and is detected in primary cultures of rat type II cells, human H441 cells, and cells transfected with a human SP-B cDNA [75,76]. The form of SP-B recovered from the airways (“mature” SP-B, or SP-B₈) is a small amphipathic peptide of 79 amino acid residues migrating at M_r 8000 in SDS-PAGE [77]. The mature SP-B form is thus contained within the sequence of a larger precursor proprotein (pro SP-B). The pro SP-B NH₂-terminal flanking region contains a classic cleavable “signal sequence,” and there are two sites in the propeptide for asparagine-linked glycosylation (Table 1). Production of SP-B₈ from the larger pro SP-B precursor involves a single cleavage of COOH flanking propeptide, followed by a two-step removal of the NH₂ flanking propeptide prior to assembly of the mature form (SP-B₈) in lamellar bodies for secretion with phospholipids [75,76,78–80]. In the alveolus, SP-B exists as a dimer linked by intermolecular disulfide bonding [75,81]. The secondary structure of SP-B has been well characterized and bears partial resemblance to members of the saposin family [82].

Surfactant Protein C

The alveolar form of SP-C (‘‘mature’’ SP-C, or SP-C_{3.7}) is composed of 33 to 35 amino acids. SP-C is a predominantly hydrophobic molecule due to a high content of Val, Ile, and Leu, which is highly alpha-helical and capable of spanning phospholipid bilayers in the liquid crystalline phase (reviewed in Refs 13 and 83). In addition to the extreme hydrophobicity conveyed by its primary sequence, mature SP-C is known to be a true lipopeptide; i.e., greater than 90% of natural bovine, canine, and human SP-C_{3.7} isolated from the surfactant of five mammalian species exists in a palmitoylated form with a palmitoyl thioester linkage to either one serine or two cysteine residues near the NH₂-terminus. This makes SP-C unique among surfactant proteins although the exact function of this modification remains currently unknown.

The full-length SP-C mRNA (0.9 kb), produced by splicing of multiple exons, yields a primary translation product 191 to 197 amino acids in length [84–86]. In vitro translation of human lung RNA produces SP-C primary translation products of M_r 21,000 to 22,000. Similar-sized products have been detected in primary cultures of rat type II cells and produced in CHO cells transfected with a human SP-C cDNA [87–92]. Like SP-B, mature SP-C is also contained within the sequence (residues 24 to 58) of a larger precursor proprotein (pro SP-C) (reviewed in Refs. 13 and 83). Unlike pro SP-B, SP-A, or SP-D, pro SP-C does not contain a classic signal sequence, and there are no sites for asparagine-linked glycosylation (Table 1). SP-C biosynthesis from the larger pro SP-C precursor is type 2 cell specific and requires a series of 4 discrete proteolytic cleavages to remove 23 to 25 residues of NH₂-terminal peptide and 133 to 139 residues of COOH-terminus, all of which occur at sites within the regulated secretory pathway distal to the trans-Golgi network [87–96]. The mature form (SP-C_{3.7}) is assembled in lamellar bodies for secretion with SP-B and phospholipids.

Structure, Function, and Biosynthesis of Hydrophilic Surfactant Proteins

SP-A and SP-D are members of a family of proteins believed to play a role in the innate or non-antibody-mediated immune response [14,27,31,97,98]. The term ‘‘collectin’’ (collagen-like lectin) has been used to describe this family, whose members outside the lung include serum proteins, mannose-binding protein (MBP), bovine conglutinin, and CL-43 (Figure 4). In addition, SP-A has structural homology to C1q. Compelling evidence for the importance of collectins to host defense comes from observations that low levels of MBP have been associated with recurrent infections in children [99,100]. Since both proteins have been shown to interact with *P. carinii* organisms [23–26,101], they now appear to

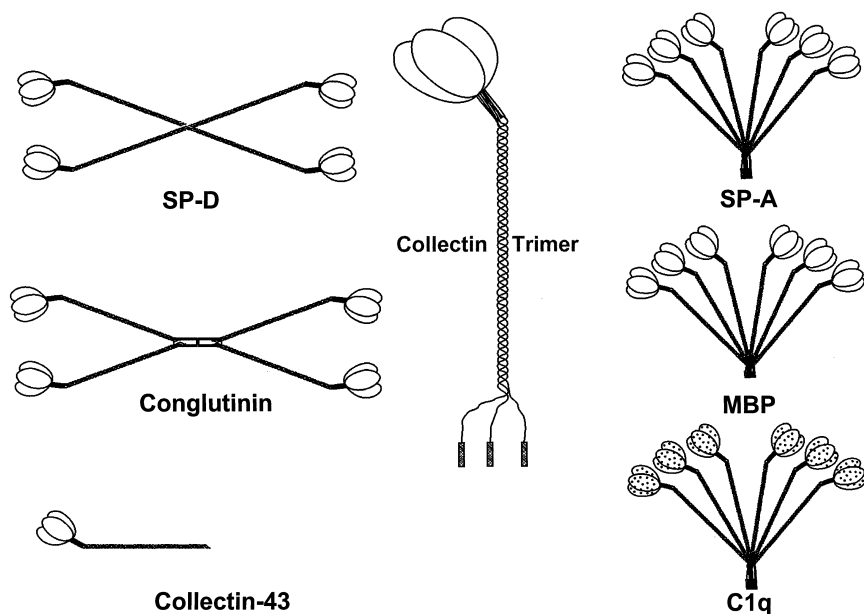


Figure 4 Multimeric structure of the collection family of C-type lectins. Collectin monomers (see Fig. 2) are assembled initially into trimeric forms (representative structure shown center) that are then organized into higher-order multimers. Based on rotary shadowing, **SP-A**, mannose binding protein (**MBP**), and complement factor **C1q** each exhibit bouquet-like structures composed of multiple trimeric units (**right**). SP-A and C1q each form octadecamers, while MBP is organized as oligomers of varying multiples (two to five trimers). In contrast, **SP-D**, as well as bovine **conglutinin**, forms dodecamers via disulfide bonding in the NH₂ terminal noncollagenous domains (**left**). Also shown in **collectin-43**, which remains predominantly in a trimeric form but bears structural and sequence similarity to the SP-D trimer.

modulate part of the increasingly complex interactions recognized to occur between host and pathogen during PcP (detailed below).

The monomeric and higher-order structures of SP-A and SP-D proteins are schematically illustrated in Figures 3 and 4.

Surfactant Protein A

SP-A, the most abundant surfactant protein, is a multifunctional sialoglycoprotein of M_r 28 to 36 kDa (reduced)[47]. In humans, there are two genes, of 4.5 kb each, located on chromosome 10 [102,103]; in mice, a single SP-A gene is localized to chromosome 14 [104]. These genes each code for a primary translation product of 255 amino acids containing a 27-amino acid cleavable signal peptide [105].

SP-A is synthesized by type II cells and nonciliated airway epithelial (Clara) cells [20]. From the cDNA sequence and structural studies, the SP-A monomeric sequence contains collagenous sequences, a COOH-terminal C-type lectin motif, a triple-helical collagen domain, and a carbohydrate recognition domain (CRD) (Fig. 3). Following synthesis, posttranslational modification of SP-A consists of N-linked glycosylation and proline hydroxylation. Subsequent to these events, assembly into higher-order multimeric forms is required. The higher-order quaternary structure of SP-A consists of an 18 mer assembled from six identical trimeric subunits linked by interchain disulfide bonding (Figure 4). The subcellular site of this assembly is presently unknown. In contrast to SP-B or SP-C, SP-A does not seem to be routed to a regulated secretory pathway but rather appears in the airspace via constitutive secretion [106], where the vast majority of the protein (> 99%) associates with phospholipid. Although SP-A can be recovered from the lamellar bodies of type II cells, it appears that it reaches this destination via an endosomal pathway following reuptake [107].

In the alveolus, SP-A binds to type II cells and macrophages in a specific, competitive manner, suggesting the presence of SP-A receptors that might augment either SP-A clearance or potentiate a signaling function [108]. Several candidate SP-A-binding proteins have recently been identified on type II cells and on macrophages [109–112].

Surfactant Protein D

SP-D is a 43-kDa monomer, which is a product of single gene also located on chromosome 10 in humans [103,113] and linked closely with the SP-A gene on chromosome 14 of the mouse [104]. SP-D protein can be detected both in type II and Clara cells [22]. In contrast to SP-A, the majority of SP-D actually partitions in the nonsurfactant portion (high-speed supernatant) of BAL fluid and does not bind surfactant phospholipid. Monomeric SP-D, like SP-A, also contains a collagen-like triple-helical domain, and a calcium-dependent lectin domain. Biosynthesis of SP-D includes posttranslational glycosylation, followed by assembly of monomeric SP-D via disulfide bonding into a dodecamer made up of four identical trimers [114] (Fig. 4). The regulation of SP-D biosynthesis and secretion is not completely defined, but like SP-A, SP-D does not appear to be routed through lamellar bodies or other regulated secretory granules.

Surfactant Collectins: A Link Between Innate and Adaptive Immunity

Evidence for the role of SP-A and SP-D in lung host defense continues to expand. Both proteins interact with a wide range of microorganisms, show specific interactions with leukocytes, and modulate the function of phagocytic cells in vitro and in vivo. Lung collectins also interact with and modulate the cellular effects of inhaled pollens and other complex organic antigens through pattern-recognition

Table 2 In Vitro Host Defense Functions of Lung Collectins

Function	Effect of SP-A	Effect of SP-D
Microorganism interactions	<i>Escherichia coli</i> (J5)	<i>Pseudomonas aeruginosa</i> (LPS)
Bacteria	<i>Haemophilus influenzae</i>	<i>E. coli</i> (rough LPS)
	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i> (LPS)
	<i>Streptococcus pneumoniae</i>	
	Group A streptococcus	
Fungi	<i>Pneumocystis</i>	<i>Pneumocystis</i>
	<i>Cryptococcus neoformans</i>	<i>Cryptococcus neoformans</i>
	<i>Aspergillus fumigatus</i>	<i>Aspergillus fumigatus</i>
Viruses	Influenza A	Influenza A
	Herpes simplex I	
	Respiratory syncytial virus	
<i>Mycobacteria</i>	<i>Mycobacterium tuberculosis</i>	<i>M. tuberculosis</i>
Leukocyte interactions	↑↑	↑↑
Chemotaxis	↑↑	↔
Phagocytosis	↑↑	↔
Organism killing	↑↑	↔
Lymphocyte proliferation	↓↓	↓↓
Free radical production	↑↑	↑↑

Abbreviations: ↑↑, Enhanced or increased; ↓↓, Inhibited or decreased; ↔, variable.

properties. These and other findings strongly suggest that SP-A and SP-D play important roles in the innate, natural, and nonclonal defense system of the lung. However, because of additional effects of these proteins on inflammatory cell activity and antigen presentation, they can, at the same time, serve as a link to adaptive immunity.

Table 2 summarizes data from a number of in vitro studies that assign function to SP-A and SP-D (reviewed in Refs. 14, 15, 27, and 97). Most of the in vitro data were obtained utilizing isolated or recombinant proteins, and both the methods of purification as well as the presence of surfactant lipids could modify some of the effects in vivo.

Lung Collectins and Pathogens

SP-A and SP-D each have been shown to interact with a variety of bacteria enhancing the selective binding and uptake by alveolar macrophages and neutrophils. Bacterial surfaces are composed of a complex array of polysaccharides and other glycoconjugates, including the polysaccharide constituents of capsules,

gram-negative lipopolysaccharides, and the lipoteichoic acid moieties of gram-positive organisms.

As can be seen in Table 2, from both in vitro and in vivo studies, both SP-A and SP-D recognize a wide variety of gram-negative and gram-positive bacteria [115–124]. SP-A has been shown to enhance not only binding but also uptake of a majority of the pathogens listed. Although SP-D binds to a variety of pathogens and induces aggregation of many, it is not as effective as an opsonin. It has been reported to enhance only modestly the phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages [125] and may, in fact, inhibit phagocytosis of *Mycobacterium tuberculosis* by macrophages [103,113,126].

The lung collectins show specific interactions with various respiratory viruses. Although the interactions of SP-D are mediated via the carbohydrate recognition domain, some interactions of SP-A with viruses require binding of viral lectins to complex sugars on SP-A. Purified SP-D and SP-A inhibit infectivity and hemagglutination activity of influenza A virus in vitro [127]. Interactions of both collectins with respiratory syncytial virus and herpes simplex virus have also been described [128–130].

The collectins also interact with fungi. Both *Cryptococcus neoformans* (unencapsulated form) and *Aspergillus fumigatus* bind SP-A and SP-D [131,132]. SP-A and SP-D also interact with *Pneumocystis*. Via its carbohydrate recognition domain (CRD), SP-A has been shown to bind to isolated *Pneumocystis* organisms in a calcium- and mannose-dependent manner [26,133,134]. Similarly, the CRD of SP-D has been shown to mediate binding of that protein to *Pneumocystis* [135]. The ligand on *Pneumocystis* to which each collectin attaches is Msg (also referred to as gpA or gp-140), the heavily mannosylated major glycoprotein found on the cell surface of both trophic forms and cysts [23,133–136]. In addition, glucans have been implicated in the interactions of SP-D with *Pneumocystis* organisms [135,137]. Chelation of calcium disrupts aggregates of organisms recovered in BAL fluid, and the isolated organisms are readily agglutinated following the addition of SP-D. Cysts and trophic forms are associated with SP-A and SP-D in the airspaces of rats and mice with PcP [138]. In vitro, SP-A has also been shown to facilitate the uptake of labeled *Pneumocystis* organisms by macrophages; however, SP-D, while increasing aggregation and adherence of the organism to macrophages, has not been shown in vitro to augment phagocytosis.

Collectin Interactions with Inflammatory Cells

In addition to interactions with specific pathogens, a wealth of in vitro data shows that both SP-A and SP-D affect a variety of immune cell functions (reviewed in Refs. 27, 103, and 113). Both SP-A and SP-D have been shown to modulate lymphocyte proliferation, macrophage activation and function, neutrophil function, and dendritic cell activity [29,139–146]. Available data suggest that the proteins elicit cell-specific responses and that, in some cases, SP-A and SP-D

can have different effects on the same cell type, depending on the state of cell activation:

1. Lymphocytes. Several studies defined a role for both SP-A and SP-D in attenuating lymphocyte responses. Both SP-A and SP-D inhibit phytohemagglutinin (PHA), and anti-CD3 stimulated T-lymphocyte proliferation in highly enriched populations of peripheral blood lymphocytes [28,29,146]. At least part of this inhibition is secondary to inhibition of production of IL-2, a potent mitogen for lymphocytes.

2. Macrophages and monocytes. Despite a common lineage, these two cell types have some shared and some markedly different responses to SP-A and SP-D. SP-A has been shown to enhance the uptake of a variety of bacteria by both alveolar macrophages and monocytes (reviewed in Table 3 and Ref. 14). Both SP-A and SP-D stimulate chemotaxis of alveolar macrophages, possibly via effects on directional actin polymerization [143]. In contrast, monocyte migration is not enhanced by SP-A. While SP-D stimulates chemotaxis of alveolar macrophages, it has been reported to either enhance [147] or have no effect [143] on monocyte chemotaxis. SP-A has been reported to variably affect cytokine production by alveolar macrophages, which are a primary source of these regulatory molecules, which play a pivotal role in the pulmonary immune response to infection and inflammation. SP-A inhibits production of cytokines in response to the inflammatory bacterial cell wall component LPS [148] and to intact *Candida albicans* [149], but it directly enhances the production of tumor necrosis factor alpha (TNF- α) by alveolar macrophages [150].

3. Neutrophils. Neutrophils respond to SP-A and SP-D. In vitro, both SP-A and SP-D enhance the phagocytosis of *Escherichia coli*, *Streptococcus*

Table 3 Immunomodulatory Functions of SP-A, SP-D, and *Pneumocystis*

Function	Effect of SP-A	Effect of SP-D	Effect of <i>Pneumocystis</i> Infection
Lymphocyte proliferation	↓	↓	↑
IL-2 production	↓	↓	??
TNF- α production (PBMC)	↑	↓	↑
TNF- α production (macrophage)	↓	↓	↑
IL-1 α production	↑	??	↑
IL-1 β production	↑	??	↑
IL-6 production	↑	??	↑
NO production (macrophage)	↑	??	↑
MMP production	??	↑	??

Abbreviations: ??, unknown; PBMC, peripheral mononuclear blood cells; MMP, matrix metalloproteinase.

pneumoniae, and *Staphylococcus aureus* by neutrophils [123]. SP-A and SP-D also enhance the phagocytosis, oxidative burst, and killing of *A. fumigatus* conidia by neutrophils [132]. In addition to enhancing neutrophil phagocytosis, SP-A and SP-D can stimulate neutrophil migration [132,147].

4. Dendritic cells. CD 11-positive immature dendritic cells show selective binding for SP-D compared to their mature antigen-presenting counterparts. Recently, in an in vitro assay, SP-D (but not SP-A) enhanced presentation of an ovalbumin peptide antigen to lymphocytes [139]. These data suggest an emerging link between innate and acquired immunity that can be mediated by collectins.

Other Effects: Cytokines and Reactive Species

The known in vitro effects of SP-A and SP-D on cytokine production and second messengers are summarized in Table 4.

The production of reactive oxygen and nitrogen species by alveolar macrophages and monocytes can also be modulated by SP-A and SP-D [151]. Both reactive oxygen and nitrogen species are important antibacterial and antiviral defenses. However, these radicals, like cytokines, have the potential to exacerbate an inflammatory response if their levels are not tightly regulated. Thus, a teleological role for collectins is the countermodulation of the proinflammatory response that develops during clearance of microorganisms or following an incendiary inflammatory event such as hyperoxia.

Lessons from Collectin Knockout Models

The role of the collectins in host defense and inflammation has been further defined in vivo in the context of the whole animal using single collectin knockout mice.

SP-A $-/-$ mice homozygous for a null mutation of the SP-A gene have been produced in the National Institutes of Health (NIH) Swiss black and noncongenic backgrounds [152,153]. Although the surfactant of these mice lacks tubular myelin, both SP-A $+/-$ and SP-A $-/-$ mice survive normally and display no abnormalities in pulmonary function. The critical role of SP-A in host defense is supported by the observation that SP-A-deficient mice show an enhanced susceptibility to infection from both group B streptococcus and *P. aeruginosa* as well as delayed clearance of influenza A virus [118,119,153,154].

Recently, SP-A $-/-$ mice have been shown to have a delayed clearance of mouse-derived *Pneumocystis* [25]. Using intratracheal inoculation as well as cohousing studies, Walzer's group has shown that SP-A $-/-$ mice develop increased cyst burden compared to wild-type controls, but immunosuppression using corticosteroids was required to achieve infection in both SP-A $-/-$ and wild-type mice.

Targeted disruption of the mouse SP-D gene has been reported by two different groups [85,155,156]. In both cases, regardless of the background strain,

the phenotype of SP-D–null mice is associated with increased alveolar lipid pool sizes. Interestingly, in addition to lipid accumulation, SP-D–/– mice, even though housed under barrier conditions, develop a proinflammatory phenotype characterized by increased BAL cellularity, activated macrophages, enhanced production of free radicals, increased release of matrix metalloproteinase (MMP), and development of premature airspace enlargement (emphysema)[157]. In vivo studies of the role of SP-D in lung host defense of specific pathogens are more limited. A role for SP-D in the augmentation of clearance and the inflammatory response to Influenza A has been shown [153,158]. No studies have been reported using these models to assess the role of SP-D in the clearance of bacteria or fungi, including *Pneumocystis*. Although the presence of SP-D is important for the modulation of the host inflammatory response, it, coupled with in vitro observations that SP-D may impair phagocytosis of *Pneumocystis*, could potentially provide a mechanism by which *Pneumocystis* organisms escape host recognition and removal.

C. Regulation of Surfactant Homeostasis

The levels of phospholipid and protein found in the extracellular pool of pulmonary surfactant appear to be tightly controlled. The syndrome of idiopathic alveolar proteinosis is associated with massive accumulation of phospholipid and protein in the alveolar space. Lipoproteinosis has been associated with some clinical presentations of PcP, and pulmonary pneumocystosis has been included in etiologies of secondary alveolar proteinosis in humans.

Metabolic Life Cycle of Surfactant Components

The metabolic life cycle of surfactant is schematically shown in Figure 5. From biochemical analysis of lamellar bodies and surfactant, it is likely that SP-B₈ and SP-C_{3,7} are stored along with synthesized surfactant phospholipid in the lamellar bodies of type II cells prior to release to the alveolar space. The effect of the known phospholipid secretagogues (discussed above) on the kinetics of SP-B/C secretion are similar, with studies showing cosecretion with the phospholipid, indicating that the same signal transduction mechanisms play a part [159]. In contrast, SP-A and SP-D are not trafficked via the regulated (lamellar body–dependent) pathway of type II cells but rather appear to reach the airways via constitutive secretion; they are therefore regulated at a different level than these other components.

The fate of surfactant proteins secreted into the alveolar space of the adult lung is relatively uncharacterized. There is a significant body of data to support the notion of reuptake of phospholipids SP-A, SP-B, and SP-C by type II pneumocytes [41,160–162]. Morphological and ¹²⁵I-binding studies in isolated type II cells confirm saturable binding, internalization, and appearance in endosomes of

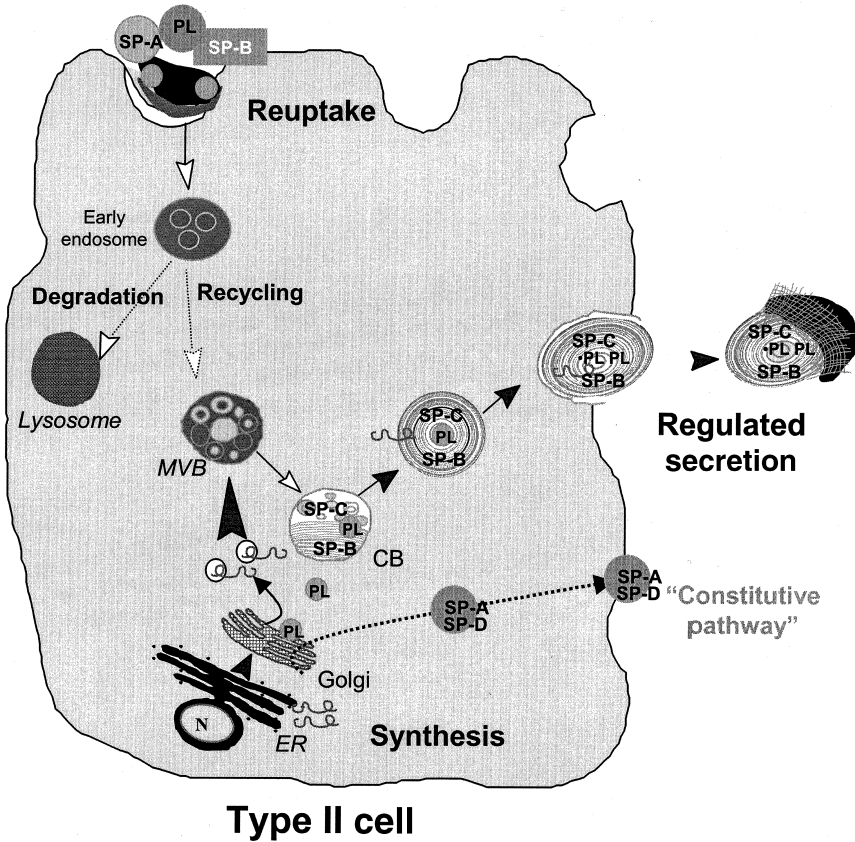


Figure 5 Schematic diagram of cellular machinery for surfactant component metabolism. Synthesis of SP-B and SP-C begins with translation of RNA in the cytoplasm. Both propeptides must be translocated to the ER, sorted in the Golgi, and then enter the regulated secretory pathway in Brefeldin A-sensitive budding transport vesicles. Major subcellular compartments include the multivesicular body (MVB), composite body (immature LB), and LB. SP-B and SP-C are secreted into the alveolar space with SP-B and phospholipid. Phospholipid synthesized de novo reaches the LB via a Brefeldin A-insensitive pathway. In contrast, SP-A and SP-D are synthesized and glycosylated during trafficking through the ER and Golgi. Unlike SP-B/C, the collectins are routed and secreted via a non-lamellar-body pathway that is not responsive to surfactant secretagogues (i.e., a "constitutive pathway"). Both phospholipids and some proteins (SP-A, SP-B, SP-C) have been shown to be endocytosed and routed back to either lysosomes for degradation or to LB via MVB. Phospholipid degradation products can also be reutilized for synthesis (not shown).

both SP-A and SP-B. Based on limited studies to date, reuptake of SP-C by type II cells also occurs, with accumulation in lamellar body-enriched fractions in a time-dependent manner [163]. Recycling of SP-D via type II cell endocytosis has not been reported.

In addition to type II cells, the alveolar macrophage has also been shown to play a role in surfactant component homeostasis. Alveolar macrophages have been shown to bind, internalize, and/or degrade phospholipid, SP-A, SP-B, SP-C and SP-D [43,164,165]. Furthermore, GM-CSF-/- mice demonstrate marked increases in the content of alveolar surfactant phospholipid, which has been attributed in part to an associated defect in macrophage phospholipid clearance in these mice [166].

The expression of surfactant components partially extends beyond the alveolar type II cell. Both in situ hybridization and immunocytochemistry have shown that the nonciliated epithelial cells of the mammalian airway (Clara cells), express SP-A, SP-D, and pro SP-B [19,20,22]. The relative amount and degree of this expression appear to vary among species, correlating with the density and location of Clara cells in the airway (mouse > human). Although little is known about the contribution of Clara cells to overall surfactant homeostasis, the spatial distribution of these cells in the distal airway suggests a potentially important role in the local regulation of collectin expression and function at this site.

Regulation of Surfactant Component Expression

The control of surfactant phospholipid and protein expression is a fairly complex event involving multiple levels of regulation. In addition to modulation at the level of synthesis and regulated secretion (reviewed in Ref. 10) and by clearance by type II cells and macrophages [9,43], the expression of individual surfactant components is also sensitive to a variety of cytokines, environmental conditions, and drugs.

As discussed in detail elsewhere in this volume, infection with *Pneumocystis* is associated with a mononuclear cell- and macrophage-dependent proinflammatory response that is required for the clearance of infection. The effective response is mediated in part via a coordinated elaboration of cytokines (both Th1 and Th2) as well as a dependence in part on other cytokines, such as TNF- α ; removal of organisms via phagocytosis and killing; a contribution from CD8+ cells; and development of an immune (antibody) response. In addition, although not well studied, through direct interaction with the organism itself, type II cell function or individual surfactant component expression may be altered. Furthermore, the injury pattern produced by PcP may be modulated by a variety of clinical situations, including concomitant use of hyperoxia or corticosteroids. Thus, during *Pneumocystis* infection, a combination of organism, host-response,

and other exogenous factors can combine to disrupt normal surfactant homeostasis. These are summarized in Table 4.

Several cytokines relevant to host defense against *Pneumocystis* have been shown to affect surfactant expression. The intratracheal instillation of TNF- α in mice is associated with time-dependent downregulation of both SP-B and SP-C gene expression [167–169]. The effects on SP-A *in vivo* are less definitive, although exogenous TNF- α will decrease SP-A expression in cultured lung epithelial cells. Data for SP-D (*in vitro* or *in vivo*) have not been reported.

The host response during PcP includes development of a Th2 cytokine response. In other systems, both IL-4 and IL-5 have been shown to affect the surfactant system. Transgenic mice overexpressing IL-4 in the lung through the use of the clara cell specific protein (CCSP) promoter develop increased levels of phospholipid and of SP-D [170]. Interestingly, these mice also show mildly decreased levels of SP-B. Recently, we have found that a single intratracheal administration of IL-4 to mice produced similar results with marked elevation of SP-D and decreased levels of SP-B 24 hr after inoculation, indicating that these are individual effects and not due to global dysfunction caused by this cytokine [171,172]. Interleukin-5 has also been implicated as a regulator of surfactant component expression. Antigenic challenge with ovalbumin of transgenic mice expressing eotaxin under the control of the SP-C promoter results in downregulation of both the eotaxin transgene as well as endogenous SP-C gene expression [173]. This effect was blocked by treatment with anti-IL-5. In contrast to Th2 cytokines, a consistent response to Th1 like cytokines (IFN- γ) has not been described.

In another model of allergic challenge using *A. fumigatus* we and others have shown the generated Th2 immune response and airway hyperresponsiveness to be associated with selective downregulation of both SP-B and SP-C, an increase in total protein in BAL fluid, and upregulation of SP-D [174]. It is clear that other forms of inflammatory and/or fibrotic lung disease may also affect surfactant homeostasis. The administration of intratracheal bleomycin is associated with the subacute development of lung inflammation over 7 to 14 days following exposure, which is associated with oxidant stress and elaboration of profibrotic cytokines (TGF- β 1). At this time, animals develop respiratory distress and have been shown to have abnormal surfactant biophysical activity, decreased levels of SP-B and SP-C, and elevations of SP-D and to a lesser extent SP-A [175]. *In vitro*, TGF- β 1 administered to cultured human fetal lung downregulates SP-A, SP-B, and SP-C gene and protein expression [176]. Taken in total, the cytokine milieu that develops during an inflammatory or infectious state can profoundly alter both intra- and extracellular surfactant homeostasis and conversely, as other findings above also suggest, surfactant can also affect the inflammatory state.

Several agents used in the treatment of PcP may have additional effects on surfactant homeostasis. Corticosteroids have been used clinically as adjuvant therapy in the treatment of severe PcP in humans to prevent mechanical ventila-

Table 4 Effect of Mediators and Conditions Known to Modulate Surfactant Component Expression

Agent	Phospholipid	SP-B	SP-C	SP-A	SP-D	Experimental Model	Reference
A. Cytokines							
TNF- α	ND	↓	↓	+/-	ND	Murine IT in vivo	168
	ND	↓	↓	↓	ND	MLE 12 cells in vitro	167
IL-4	↑	↑		↑	↑	Transgenic mice (overexpressor)	169
	ND	↓		ND	↑	Murine IT in vivo	170
IL-5	ND	↔	↓	↔	↑	Transgenic Mouse/IL-5 neutralization	173
TGF- β_1	↓	↓	↓	↓	ND	Cultured human fetal lung in vitro	176
IFN- γ	ND	↔	↔	↑	ND	Cultured human fetal lung in vitro	208
B. Exogenous factors							
Glucocorticoids	↑	ND	ND	↑	ND	Rat in vivo	177
Hyperoxia	ND	↑	↑	↑	ND	Rat in vivo	209
Hyperoxia	↑	↓	↑	↑	ND	Hamster in vivo	210
Hyperoxia	ND	↑	↔	↑	ND	Rabbits in vivo	211
Bleomycin injury	-	↓	↓	↑	↑	Mice in vivo	175
ARDS	↓	↓	ND	↓	ND	Humans	193
Antigen challenge	↔	↓	↓	↑	↑	Mice in vivo	174

Abbreviations: IT, intratracheal administration; ND, not done (or unknown); ↔, no change; ARDS, adult respiratory distress syndrome.

Table 5 Effect of PcP on Surfactant Protein and mRNA Expression

Component	Protein		mRNA	
	Uninfected	PcP	Uninfected	PcP
SP-B	100%	45%	100%	30–35%
SP-C	100%	18%	100%	26%
SP-A	100%	344%	100%	94%
SP-D	100%	350–377%	100%	332%

Sources: Data for normalized SP protein and mRNA contents were modified from Refs. 35, 138, and 184. For comparison, all results are expressed as a percent of value for uninfected mice with ranges shown if derived from multiple reports.

tion, and doses are used to generate immunocompromised animal models of *Pneumocystis* infection. Two separate reports have demonstrated that administration of dexamethasone to adult rats increased the phospholipid pool and SP-A levels in alveolar surfactant [177,178]. Limper et al. have shown that in the corticosteroid-treated rat model of *P. carinii* pneumonia, SP-D content of surfactant increased in the control (uninfected) animals [179]. In contrast, Dichter et al. reported that administration of adjuvant methylprednisolone to HIV-infected patients with PcP did not alter the rate of recovery of surfactant lipid levels after 10 days of therapy [180]. However, both in human fetal lung preparations cultured in vitro and in human fetuses of mothers in preterm labor in vivo, administration of steroids have been shown to have an effect on the regulation of all four surfactant proteins and lipids [181–183]. Therefore steroid administration can be a confounding variable in the interpretation of studies of surfactant component expression, and the changes observed may provide a mechanism for the observed benefit of adjuvant steroids in moderate to severe PcP.

PcP is almost always accompanied by a requirement for supplemental oxygen therapy. Prolonged exposure to high concentrations of oxygen is well known to affect several aspects of the surfactant system. In addition to inactivation of surfactant biophysical activity from protein leak, several surfactant components are altered during the development of O₂ injury (Table 4). However, the specific effects of hyperoxia are partly species- and dose-dependent, and any effects upon surfactant protein expression, phospholipid synthesis, secretion, or recycling directly attributed to oxygen must be interpreted against the background of general cellular toxicity often seen with such exposures.

III. Alterations in Surfactant Component Expression Induced by *Pneumocystis* Pneumonia

Multiple abnormalities in surfactant composition and function have been described in PcP in humans and through the use of both in vitro systems and a

variety of immunocompromised animal models [16,17,180,184]. The major quantitative changes are summarized in Table 5. As mentioned previously, prolonged corticosteroid administration could mask the characterization of specific *Pneumocystis*-induced changes in surfactant in models relying on this modality to achieve immunosuppression required for infection. Furthermore, in humans, the effects of CD4 cells and HIV infection on the surfactant system has not been completely characterized and could represent confounding variables.

A. Changes in Lipid Composition and Biophysical Activity

Early observations focused on the characterization of phospholipid metabolism and function. Decreases in total phospholipid content and alterations in relative proportions of individual lipid components (PG, DPPC, lysoPC) were noted in surfactant from both humans and experimental animals with PcP [16,35, 138,180,184–187]. In a rat model of PcP, infection resulted in decreases in PG and in alterations in the minimum surface tension of surfactant recovered from the BAL fluid [187]. Other surfactant components were not assessed in this study. In humans, analysis of BAL has also demonstrated significant decreases in both total phospholipid levels and in lipid subtypes [16,17]. Elevated phospholipase activity has also been described [16]; the significance of this finding is unclear, but it could account for the increase of lysoPC reported by some investigators.

While expression and regulation of enzymes and pathways responsible for synthesis of surfactant phospholipid have not been assessed in PcP, at least part of the alterations can be accounted for by changes in secretion of phospholipids. Type II cells isolated from corticosteroid-treated rats infected with *P. carinii* demonstrate decreased basal and stimulated secretion, indicating that *Pneumocystis* can affect the regulated secretory pathway of type II pneumocytes [188]. In vitro, pretreatment of cultured type II cells isolated from uninfected Sprague-Dawley rats with the purified major surface glycoproteins of *P. carinii*, Msg, resulted in complete inhibition in basal phospholipid secretion and significant blunting of the response to the surfactant secretagogue ATP [189]. These results suggest that direct interaction of type II cell with the *Pneumocystis* organisms or with shed Msg can alter surfactant homeostasis. However, given the magnitude of the observed decreases in lipid pool sizes (50%), in lipid subtype composition, and in secretion, phospholipid homeostasis alone is likely insufficient to explain the profound physiological alterations seen during PcP and implies either a deficiency of surfactant protein components or inactivation of biophysical activity by exogenous inhibitor (s).

B. Alterations in Expression and Distribution of Surfactant Components

More recently, analysis of surfactant component expression in PcP has relied on the use two immunocompromised models of infection. The severe combined

immunodeficiency (SCID) mouse model, based on the original C.B-17 congenic strain, has greatly reduced numbers of T and B cells and lacks circulating immunoglobulin. *Pneumocystis* lung infection can then be deliberately initiated by intratracheal inoculation of organisms. The appearance of significant pneumonia occurs within 2 to 6 weeks. A second mouse model of PcP has been described using monoclonal antibodies to selectively deplete helper T lymphocytes in virus-free mice that are then given *P. carinii* f.sp. *mus* via intratracheal inoculation. Although it requires the interval parenteral administration of depleting antibody, the model is complementary to the SCID model and may more accurately mimic the clinical scenario associated with advanced HIV infection. Using these models (which do not require corticosteroids), the selective effects of PcP on each major component of the surfactant system have been determined.

Table 5 summarizes the effects of *Pneumocystis* pneumonia on surfactant protein expression obtained from several studies [35,138,184,190]. The development of *Pneumocystis* infection in SCID mice 4 to 6 weeks after inoculation of *Pneumocystis carinii* results in greater than 50% decrease in total phospholipid and significant loss of SP-B and SP-C protein from the LA surfactant. In this model, PcP-induced decreases in hydrophobic surfactant protein levels measured are associated with a loss of biophysical surface activity. Because surface tension assessment measured in vitro utilized equal phospholipid concentrations, the loss of biophysical activity cannot be explained by differences in total phospholipid levels. The magnitude of the decrease in SP-B observed was similar to that for heterozygous SP-B knockout mice [191] and in sepsis patients shown to be at risk for ARDS [192,193]. The level of SP-C in these two models is also reduced. Extremely low or absent levels of SP-C in association with a 50% reduction in SP-B were reported in Belgian calves dying of respiratory failure suggested that the observed decreases are physiologically relevant [68].

Importantly, in contrast to the hydrophobic proteins, *P. carinii* induced differential expression of the lung collectins SP-A and SP-D, which included a marked upregulation of SP-D protein in both the SA and LA fractions [35,138]. Immunohistochemistry demonstrated that in PcP, the upregulation of SP-D was localized to regions of atelectasis and cellular infiltration that contain *P. carinii* organisms. The SP-A protein content of LA surfactant and lung tissue remains unchanged under these conditions but is increased in SA fractions, leading to an increase in total alveolar SP-A: In a corticosteroid-treated rat model of PcP, both SP-A levels and SP-D levels have been reported to be increased [178,179]. Similarly, BAL fluid from PcP in HIV-infected patients also showed increases in SP-A [194,195]. Of interest, clusters of *Pneumocystis* organisms were noted in the BAL fluid following low-speed centrifugation. Since SP-A has been shown to bind to *Pneumocystis*, it is possible that organism-associated SP-A contributed to the elevation of levels in those studies.

Taken in toto, in murine and rat models of PcP and from limited clinical data from humans with PcP, *Pneumocystis* infection in the lung induces selective alterations in surfactant component expression. The findings collectively indicate that there is an absolute decrease in hydrophobic components (SP-B, SP-C, and phospholipid), an absolute increase in lung SP-D, and a redistribution of SP-A and phospholipid to the small aggregate (hypophase) fraction following *Pneumocystis* lung infection. Therefore the observed changes induced by PcP are selective and unlikely to be due to nonspecific alveolar damage as is seen in ARDS or in chronic lung injury, such as bronchopulmonary dysplasia.

C. Mechanisms for Surfactant Protein Changes: Gene Expression, Biosynthesis, and Secretion

The mechanisms governing the differential expression of the hydrophobic proteins SP-B and SP-C, SP-D, and SP-A during the development of PcP are incompletely defined but appear to occur at many levels, consistent with the known complexities underlying surfactant protein expression, secretion, and recycling.

In two studies using a SCID mouse model, the relative levels of expression of SP mRNA during PcP were quantitated. Table 5 summarizes data for surfactant mRNA expression in this model in comparison to protein expression and phospholipid levels. The data indicate a differential regulation of the four surfactant proteins occurs in response to *Pneumocystis*. PcP significantly downregulated levels of SP-B and SP-C protein with commensurate changes at the mRNA level. Similarly, total alveolar SP-D protein and mRNA levels were increased in a parallel fashion also suggesting transcriptional regulation of this surfactant protein. Levels of SP-B, SP-C, and SP-D mRNA expression are known to be altered by a variety of agents including hormones (dexamethasone, insulin) and second messengers (cAMP, phorbol ester) (see Table 4). In contrast to the hydrophobic proteins and SP-D, the elevation in total alveolar SP-A protein observed with *P. carinii* infection occurs without changes in mRNA expression, suggesting that this is occurring downstream from transcriptional events. Thus, the differential regulation of mRNA for these four components likely represents a complex interaction between several mediators present in the inflammatory milieu induced by PcP.

As mentioned previously, secretion of surfactant phospholipid by type II cells is inhibited by the major surface glycoprotein of *Pneumocystis* [189]. Since it is known that both SP-B and SP-C are also assembled into lamellar bodies and cosecreted with phospholipids [159], it is likely that, in addition to downregulation of hydrophobic protein biosynthesis, the effect of Msg on regulated secretion of type II cells may also play a role in modulation of these levels. The biosynthesis of SP-D is incompletely understood, but its release into the alveolar space is not felt to be lamellar body-dependent and thus unaffected by Msg.

As for the protein expression, in contrast to SP-B, SP-C and SP-D, the elevation in total alveolar SP-A protein observed with *P. carinii* infection occurs without changes in mRNA expression, suggesting that this is occurring downstream from transcriptional events. Based on the known biosynthetic pathway and properties of SP-A, there are several potential mechanisms. Previously, we made a similar observation of the regulation of SP-A biosynthesis in response to secretagogue treatment. In an isolated perfused rat lung model, treatment with either 8-Br-cAMP or ATP stimulated SP-A protein synthesis, but without detectable changes in mRNA, consistent with translationally mediated regulation [196]. In type II cells, following translation, SP-A is known to be constitutively secreted via a non-lamellar-body pathway [106,197]. Therefore elevations in small aggregate SP-A could also be the result of continued constitutive secretion and redistribution of SP-A into the SA fraction that is insensitive to factors affecting feedback inhibition of the regulated secretory pathway and not dependent of changes (increases) in SP-A mRNA expression. In addition to translational modulation of SP-A biosynthesis and secretion, increases in collection expression observed in PcP could also be attributed to an acquired impairment of surfactant protein clearance, since *P. carinii* binds both SP-A and SP-D and the collectins promote aggregation of the organism *in vitro*. Immunohistochemistry of lungs from mice with PcP has suggested that SP-A and SP-D accumulate at sites of alveolar *P. carinii* elaboration [23]. A large burden of organisms could act as an extracellular reservoir for the accumulation of surfactant proteins during pneumonia. Thus, the total amount of SP-A in the BAL fluid may be increased as a result of a combination of increased translation, continued constitutive release, and decreased clearance.

D. The Contribution of Host Inflammation to Surfactant Dysfunction During *Pneumocystis* Pneumonia

It is known that in PcP—as well as in other forms of lung infection, acute lung injury, and inflammation—BAL fluid demonstrates significant increases in total protein content as well as alterations in surfactant lipid subtypes (increased lysoPC, decreased PG). Through analysis of surfactant obtained from both animal models and humans with these injury patterns as well as through *in situ* biophysical measurements, it is well established that the surface activity of lung surfactant can be modulated by a variety of inhibitors known to be elaborated during these inflammatory processes [198–200]. The presence of inhibitors in surfactant was first shown by studies in which elevated surface tension measurements present in surfactant from these injury models could be corrected by extraction of the phospholipid/protein, thus indicating the presence of an exogenous inhibitory substance. In some cases, the inhibitory effects can be overcome by the addition

of more lipid or surfactant protein components, indicating that a deficiency relative to the amount of inhibitor is present.

Though a variety of in situ studies using purified protein derivatives, a number of protein components—in most cases normally present in serum—have been shown to promote surfactant inactivation. Notable among these inhibitors are fibrinogen, serum proteins (including albumin), and lysophosphatidylcholine, which accumulate during *P. carinii* lung infection [16,138,201].

Wright et al. have utilized both *Pneumocystis*-infected SCID mice and selectively T cell-depleted mice to assess the role of pulmonary inflammation in lung dysfunction resulting from PcP [190,202]. SCID mice and mice selectively depleted of both CD4 and CD8 cells develop PcP following intratracheal inoculation with organisms, but they exhibit few signs of inflammation and display normal surfactant biophysics. When *Pneumocystis*-infected SCID mice are immunologically reconstituted with CD8 cells, pulmonary inflammation develops (as manifest by increased protein leak) and is associated with elevated surfactant surface tensions. In these studies, SP-B mRNA was moderately decreased; however, neither SP-B nor SP-C protein levels were measured.

Taken in total, the alteration in lung mechanics and gas exchange present in PcP is likely due to altered surfactant biophysical activity resulting from the interplay of multiple mechanisms, including alterations of lipid subtypes, decreases in essential hydrophobic surfactant proteins B and C, increases in exogenous inhibitors, and increased susceptibility of the LA surfactant to inactivation by these inhibitors.

IV. Summary and Conclusions

It has become increasingly apparent that during the pathogenesis of PcP that the surfactant system plays important role in the modulation of lung mechanics/gas exchange, in the mediation of the innate immune response to the organism, and in the modulation of the host inflammatory response required for effective clearance. The changes in surfactant component expression and biophysical activity represent a complex pathophysiology involving interactions between many pathways and mediators. Based on the data to date and the known cell biology of surfactant metabolism and function, a working model can be elaborated, which is illustrated in Figure 6. Collectively, these data indicate that PcP induces selective downregulation of SP-Bs and SP-Cs, with corresponding changes in surface tension. In addition, the host response can further modulate this activity through inhibition of surfactant activity.

The alterations in hydrophobic protein content raise the possibility that surfactant replacement could be useful as adjunctive therapy in PcP. Hughes et al. have shown the efficacy of exogenous surfactant replacement in a rat model of PcP [203]. In humans, this has been attempted on a limited scale, with several

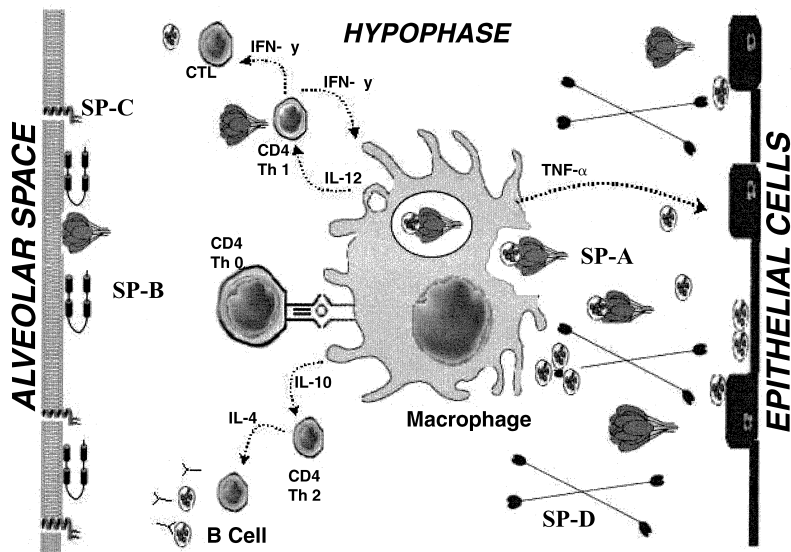


Figure 6 Model for surfactant component expression, function and modulation during PCP. Direct attachment of *Pneumocystis* to alveolar epithelia (type I greater than type II cells) establishes infection (206, 207) and leads to cell damage, production of inflammatory mediators, and upregulation of collectin (SP-A and SP-D) production by type II and Clara cells. A well-characterized and coordinated T-lymphocyte response develops in which the multipotential T lymphocyte is activated and proliferates activating both Th1 and Th2 pathways, resulting in secretion of IFN- γ (Th1) and IL-4 (Th2). SP-A binds to *Pneumocystis* further increasing its local alveolar concentration, whose effects include but are not limited to (1) stimulation of proinflammatory cytokine production by macrophages, which include TNF- α ; (2) enhanced binding and phagocytosis (i.e.) clearance of organism) by alveolar macrophages; (3) generation of reactive species (NO, O₂) 3 and (4) elaboration of additional cytokines. Free SP-D and SP-D bound to PC can also augment free radical production by macrophages for killing. Three host responses are possible: (1) In **normal hosts**, these events result in killing of the organism, *Pneumocystis* is cleared, and inflammation/lung injury limited by a balance between pro- and anti-inflammatory effects of SP-A and SP-D on both alveolar macrophage and lymphocytes. (2) In **immunocompromised hosts**, clearance of *Pneumocystis* based on collectins alone (innate immunity) ultimately fails, but proinflammatory cytokine and mediator production continues via macrophage and CD8 cells. The burden of *Pneumocystis* produces alterations in surfactant lipid secretion, modulation of surfactant protein gene transcription, changes in surfactant protein production and altered surface tension and lung mechanics. (3) In hosts deficient in SP-A or SP-D, (e.g., **knockout mice**), despite a full T-cell repertoire, alveolar macrophages are inefficient at presenting antigen or clearing the burden of *Pneumocystis*. Persistence of infection will occur and enhanced inflammation will develop late as a consequence of the imbalance between levels of SP-A, SP-D, and proinflammatory events.

case reports and a small series showing some benefit [204,204,205]. The role of altered surfactant composition and biophysical function cannot be underestimated, and the recognition of these changes raises the possibility of surfactant as a new therapeutic intervention in the treatment of this infection.

Furthermore, the selective upregulation of SP-Ds and SP-As raises new questions for the role of lung collectins in the host-defense and inflammatory response to this opportunistic infection. Future investigations focused on lung collectins and their interactions with microbes and host cells may provide further insights for prevention and treatment of *Pneumocystis* pneumonia and other infections of the lower respiratory tract.

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14

Mechanisms of Lung Injury During *Pneumocystis* Pneumonia

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I. Introduction

Although significant progress has been made in our understanding of *Pneumocystis* pneumonia (PcP), the exact mechanisms of the associated lung injury remain largely undetermined. The lack of a reliable culture system for this organism has hampered the identification and characterization of *Pneumocystis*-specific gene products that are directly involved in damaging the lung. However, more efficient identification and greater understanding of the *Pneumocystis* gene products that contribute to lung injury should be facilitated by the ongoing *Pneumocystis* Genome Project. In addition, there are several good animal models of infection that closely emulate the pathological process observed in humans with PcP. These models have been employed successfully to acquire valuable information about the host-parasite interaction as well as the lung injury and respiratory impairment resulting from that interaction. This chapter discusses important observations made in patients with PcP, as well as direct experimental data obtained from controlled animal studies, that have contributed to relevant discoveries related to the lung injury associated with *Pneumocystis* pneumonia. Together, these clinical and experimental findings have helped explain the clinical features observed in patients with PcP, and have aided in the clinical treatment of this syndrome.

Early studies of corticosteroid-treated rats determined that *Pneumocystis* organisms attach specifically to type I alveolar epithelial cells and can cause degeneration of these cells in the absence of significant inflammatory changes (15). Notably, these studies were performed in severely immunosuppressed rats that gradually acquire *Pneumocystis* from the environment without active inoculation. Therefore the direct effects of *Pneumocystis* on the architectural integrity of the lung were examined under conditions where it would be unlikely that the inflammatory contributions of advanced PcP would be observed. As a result, the role of inflammation in the pathophysiology of PcP was underappreciated. In contrast, clinical descriptions of PcP in human patients were, by nature, on a more advanced stage of disease and showed more obvious inflammatory involvement [6,13]. Subsequent animal studies confirmed that, under certain clinically relevant conditions, inflammatory lung changes are evident with advancing disease [14,18]. Therefore it appears that in addition to direct damage by the organism, *Pneumocystis*-stimulated inflammatory lung injury also plays an important role in the pathophysiology of PcP.

The clinical setting in which PcP is manifest must also be considered in discussing immune or inflammatory-mediated vs. direct *Pneumocystis*-mediated lung injury. Since lung injury during PcP is most likely a combination of the direct effects of the organism and the host's response to the organism, the immune status of the host is an important determinant of overall lung injury and the ultimate outcome of the infection. While all patients that develop clinical PcP are immunosuppressed, the predisposing conditions for PcP likely vary with regard to their impact on the immune-mediated inflammatory response. Patients with greater immune competency may better control organism numbers but suffer greater inflammatory injury. Conversely, patients with more severe immunosuppression may suffer fewer inflammatory complications but be more susceptible to direct effects of higher organism burdens. These issues are discussed in greater detail in the sections below.

II. Direct Effects of *Pneumocystis* on Lung Injury

Based on the examination of human autopsy specimens, it was originally hypothesized that PcP-associated respiratory failure was caused by disruption of gas exchange by the filling of the alveoli with organisms [19]. Subsequent studies indicated that specific early changes occurred at lower numbers of *Pneumocystis*, thereby demonstrating that the pathophysiology of PcP is far more complex than simple asphyxia caused by physical obstruction of the alveoli. Ultrastructural observation of the progression of *Pneumocystis carinii* pneumonia (PcP) in steroid-treated rats and ferrets revealed that the first noticeable event in the infectious process is the attachment of *Pneumocystis* trophic forms to the type I pneumocyte [1,3,14,20]. This is followed by increased permeability of the alveolar-capillary

membrane, degeneration and focal necrosis of the type I pneumocyte, and denudement of the basement membrane [1,3]. Importantly, these early changes occurred in the absence of a noticeable inflammatory response. Proliferation of type II pneumocytes is also observed, presumably in an effort to repair the alveoli by replacing the destroyed type I cells [1,5]. There have also been reports of increased alveolar permeability and pulmonary deficits during the early stages of infection in human patients [21,24]. Because these pathological changes occurred without a pronounced interstitial infiltrate, the suggestion has been made that direct *Pneumocystis*-mediated, inflammation-independent mechanisms of lung injury exist.

A. Effect of *Pneumocystis* on Alveolar Epithelial Cells

The close interaction of *Pneumocystis* with the type I pneumocyte was one of the first observations offering insight into the pathogenesis of PcP. Consequently this interaction and the effect it has on the pneumocyte has been extensively studied. Electron microscopic analyses of *Pneumocystis*-infected lungs from both humans and experimental animals have demonstrated the direct attachment of *Pneumocystis* to the type I pneumocyte. Although there is no evidence of host-parasite cell membrane fusion, filopodia and tubular projections extending from the organism to the type I pneumocyte have been described [5]. An in situ adhesion assay has also been used to demonstrate the specific attachment of purified *Pneumocystis* to type I pneumocytes in formaldehyde-fixed rat lung sections [25]. In addition, several in vitro studies have described both direct and ligand-mediated attachment of *Pneumocystis* to pulmonary epithelial cells [26,30]. Fibronectin, vitronectin, and laminin have been described as bridging molecules linking *Pneumocystis* to the epithelial cell [27,31,32]. The attachment of *Pneumocystis* to the type I pneumocyte likely has consequences for both direct *Pneumocystis*-mediated lung damage and also for the initiation of subsequent immune and inflammatory mechanisms of injury.

Careful studies in animal models have demonstrated that the interaction of *Pneumocystis* with the epithelium leads to degeneration of the type I pneumocyte, denudement of the basement membrane, and capillary leak. Although the exact mechanisms leading to this phenomenon are unknown, several studies have indicated that *Pneumocystis* may directly alter the pneumocyte. *Pneumocystis* attachment activates the plasmalemmal vesicular system in type I pneumocytes of corticosteroid-treated rats [33,34]. While activation of this physiologically important pathway may serve as a mechanism by which the organism acquires nutrients, it may also lead to as yet undetermined but pathologically important changes in the type I pneumocyte. It has been suggested that activation of the plasmalemmal vesicular system may be partly responsible for the alveolar leakage associated with PcP [34]. Despite these findings, it should be noted that at least one study has found that *Pneumocystis* does not directly disrupt the barrier function of

primary rat alveolar epithelial cell cultures [35]. Instead, the authors of the study suggested that additional inflammatory signals may be required to mediate the *Pneumocystis*-induced epithelial changes characteristic of PcP.

Although not as common as binding to type I pneumocytes, the adherence of *Pneumocystis* to rat type II pneumocytes has been reported [5]. Whether preferential binding to type I cells is simply related to the disproportionately large surface area they cover in the alveoli (~96%) or if this tropism is inherent to the biology of *Pneumocystis* is unknown. However, in vitro studies have demonstrated direct and indirect interactions between *Pneumocystis* and type II pneumocytes. The direct contact of high concentrations of *Pneumocystis* with monolayers of primary type II pneumocytes has been reported to cause type II cell degradation [36]. Limper and Martin extended these observations and determined that purified rat *Pneumocystis* adheres to the human adenocarcinoma A549 cell line and inhibits its growth [26]. Ensuing studies used a mink lung epithelial cell line to demonstrate that *Pneumocystis*-mediated effects on epithelial cell proliferation proceed via inhibition of cyclin-dependent kinase activity [37]. Ultimately, the interaction of *Pneumocystis* with type II pneumocytes may contribute to the pathophysiology of PcP by preventing the proliferation and migration of these cells to repair the damaged basal lamina.

The aforementioned studies provide evidence that *Pneumocystis* can exert direct effects on both type I and II pneumocytes. The attachment of *Pneumocystis* to type I pneumocytes may directly injure the alveolar epithelium or at least facilitate injury to it. Subsequent effects on type II cells may exacerbate lung injury by preventing the proliferation and migration of these cells to repair the initial damage. Additional studies are required to determine the specific contribution that these mechanisms of injury make to overall respiratory impairment during PcP. In addition to direct attachment-mediated alteration of the alveolar epithelium, *Pneumocystis* may also exert attachment-independent effects on epithelial cells. *Pneumocystis*-stimulated alveolar macrophages release cytokines that can influence gene expression in type II pneumocytes [38], and *Pneumocystis*-derived proteases and glycolytic enzymes may also contribute to epithelial damage. Furthermore, the increasingly recognized role of alveolar epithelial cells as important sources of proinflammatory mediators must also be considered. For example, it has been reported that alveolar epithelial cells secrete proinflammatory cytokines—including IL-6, IL-8, and MCP-1—following stimulation with *Pneumocystis* [39,40]. It is possible that these mediators act locally within the microenvironment of the alveoli and contribute indirectly to the observed *Pneumocystis*-mediated effects on epithelial cells.

B. *Pneumocystis*-Derived Proteolytic and Glycolytic Enzymes

The lack of a reliable culture system to propagate purified quantities of *Pneumocystis* has hampered the identification and characterization of *Pneumocystis*-

specific products that may directly contribute to lung injury. Despite this obstacle, several potential virulence factors of *Pneumocystis* have been identified. It is possible that the primary functions of the enzymes discussed here are the acquisition of nutrients or the evasion of host defense mechanisms. However, these proteins may also produce collateral damage to the lung while performing their intended functions in the life cycle of *Pneumocystis*. In addition to the specific examples discussed here, it is anticipated that the list of *Pneumocystis* gene products having the potential to inflict lung damage will grow as more of the *Pneumocystis* genome is elucidated.

Enhanced protease activity has been detected in the lung during PcP, and at least some of this activity can degrade components of the pulmonary extracellular matrix. Although host-derived proteases may contribute to the total detectable activity in the infected lung, there is also evidence suggesting that a number of these proteases are derived from *Pneumocystis*. It has been reported that *Pneumocystis* has chymase activity that is capable of compromising the alveolar-capillary barrier [41]. In addition to increased host matrix metalloproteinase (MMP) and serine protease activity during PcP, a novel collagenase activity, possibly *Pneumocystis*-derived, was also present in the lungs of infected rats [34]. Using cultured rat *Pneumocystis* to minimize host contamination, it was determined that *Pneumocystis* possesses both protease and elastase activity. The authors suggested that these enzymes are critical for proliferation of *Pneumocystis*, because a protease inhibitor, leupeptin, demonstrated anti-*Pneumocystis* properties in vitro [42]. The existence of *Pneumocystis*-derived cysteine proteases has also been reported. A 68-kDa cysteine protease capable of digesting collagen and fibronectin was found in rat *Pneumocystis* [43]. Cysteine protease activity was also detected in the lungs of *Pneumocystis*-infected rats by zymography. These bands of protease activity were also present in purified *Pneumocystis* lysates and disappeared with clearance of *Pneumocystis* from the lung following antimicrobial treatment [44]. These studies suggest that *Pneumocystis* possesses specific proteases that are capable of degrading components of the pulmonary interstitium. The potential role of these proteases in contributing to lung injury related to PcP remains undetermined.

Fungal kexin-like molecules have also been identified in mouse-, rat-, and human-derived *Pneumocystis* [45,48,49]. Single-copy genes encode the kexin protein in mouse- and human-derived *Pneumocystis*, but multiple genes encode a family of kexin-like proteins in rat-derived *Pneumocystis*. Although fungal kexins are typically associated with the processing of protein precursors in the Golgi apparatus, the mouse- and rat-derived *Pneumocystis* kexin has been localized to the surface of the organism. The importance of this in relation to lung injury is as yet undetermined. However, it has been suggested that the presence of functional kexin activity on the surface of *Pneumocystis* may play a role in the host-parasite interaction. By altering or inactivating host proteins, kexin may

facilitate attachment, nutrient acquisition, or evasion of host defense. Consequently, it may also contribute to lung injury related to PcP.

Recently, the gene encoding the glycolytic enzyme enolase was cloned from rat-derived *Pneumocystis* and characterized [50]. *Pneumocystis* enolase demonstrated significant homology to other fungal enolases. However, unlike other fungal enolases, recombinant *Pneumocystis* enolase demonstrated plasminogen-binding activity. Increased fungal enolase has been associated with invasive candidiasis, and the plasminogen-binding activity of enolase from group A streptococci has been implicated in tissue invasion [51,52]. Thus, the plasminogen-binding activity of *Pneumocystis* enolase may alter the fibrinolytic response in the lung and provide another mechanism by which *Pneumocystis* can generate tissue damage.

Several candidate molecules have been identified that could potentially contribute to *Pneumocystis*-mediated lung injury. In addition to representing therapeutic targets to alleviate lung injury during PcP, these molecules may also represent potential vaccine candidates to prevent infection.

C. *Pneumocystis*-Mediated Disruption of the Pulmonary Surfactant System

The effects of *Pneumocystis* on pulmonary surfactant have been widely studied. *Pneumocystis*-related changes in surfactant protein and phospholipid composition have been described in both humans and animal models of disease [24,53,63]. In addition, direct surface activity measurements of the cell-free lavage collected from *Pneumocystis*-infected rats and mice confirms that these compositional changes are physiologically relevant and associated with reduced surface activity [58,62,64]. Furthermore, anecdotal reports of surfactant replacement therapy have suggested some efficacy in correcting these PcP-related surfactant deficiencies in both clinical and laboratory settings [65,69]. Together, these findings indicate that changes in surfactant homeostasis play an important role in respiratory impairment during PcP.

Although the exact role of *Pneumocystis* in directly mediating surfactant disruption has not been well characterized, there are data demonstrating that *Pneumocystis* can directly alter surfactant in the absence of significant inflammation. In vitro studies have demonstrated that *Pneumocystis* and *Pneumocystis*-derived proteins inhibit surfactant protein and phospholipid secretion from alveolar epithelial cells [70,71]. In addition, *Pneumocystis* has been shown to bind surfactant components and possibly sequester them from active surfactant aggregates [36,72,75]. Furthermore, studies of early *Pneumocystis* infection in AIDS patients and animal models also suggest that inflammation-independent effects on surfactant are evident. Phosphatidylglycerol deficiencies were detected in the bronchoalveolar lavage samples from *Pneumocystis*-infected AIDS patients

before the onset of overt clinical symptoms of PcP [24]. Similar results were found using steroid-treated rats with light to moderate infections [58]. Surfactant deficiencies were also detected in *Pneumocystis*-infected mice with severe combined immunodeficiency (SCID), which are severely impaired in their ability to mount immune and inflammatory responses against *Pneumocystis* [62,64]. These studies suggest that *Pneumocystis* can exert direct negative effects on pulmonary surfactant by inhibiting the secretion of surfactant components from type II alveolar epithelial cells or by binding to specific surfactant components and sequestering them in inactive forms. A more detailed discussion of the surfactant system is provided elsewhere in this book.

III. Immune-Mediated Inflammatory Lung Injury

The differential manifestation of PcP in various clinical settings suggests that lung injury is not simply a function of organism burden but that host factors are also involved in the pathogenesis of this disease. In particular, a role for inflammatory mechanisms of lung injury has been indicated. In cancer patients, the initial observation of PcP is commonly noted when anti-inflammatory and immunosuppressive corticosteroid therapy is being tapered [76,77]. In bone marrow transplant recipients, the clinical onset of PcP is typically not evident until after engraftment and restoration of immune function, despite the fact that these patients are at risk for PcP from the time of transplant or even earlier [78,83]. The generation of glomerular basement membrane autoantibodies, which have the potential to cross-react with the alveolar basement membrane, has also been documented in AIDS patients with PcP. This may represent another mechanism by which the host response can directly contribute to lung injury related to PcP [84]. Evidence of inflammatory involvement is also demonstrated by the fact that increased bronchoalveolar lavage fluid IL-8 and neutrophil concentrations, but not organism number, correlate with decreased oxygenation, greater risk of mechanical ventilation, and increased mortality in PcP patients [85,93]. In addition, an increased serum level of type III procollagen peptide is a negative prognostic factor during AIDS-related PcP and correlates with decreased arterial oxygenation and increased risk of mechanical ventilation and death [94]. Finally, the beneficial effects of adjunctive corticosteroid therapy on lung injury during moderate to severe PcP in both non-AIDS and AIDS-related PcP has been well documented [95,101]. Presumably, the broadly anti-inflammatory and immunosuppressive properties of these compounds are responsible for respiratory improvement, again suggesting that inflammation is an important component of lung injury during PcP.

The lung-specific production of proinflammatory mediators during PcP is also well supported by the literature and may represent a mechanism by which inflammatory lung injury is generated. Increased levels of proinflammatory cyto-

kines, including TNF- α and the IL-8-related molecules, have been measured in the lungs of both patients and laboratory animals infected with *Pneumocystis* [17,89,90,102,105]. More specific in vitro studies have demonstrated that direct stimulation of alveolar macrophages or epithelial cell lines with *Pneumocystis* induces cytokine and chemokine secretion [39,40,103,106,110]. Furthermore, elevated activity of specific host MMPs have been detected in the lungs of steroid-treated rats with PcP [18,34], and gene expression of the acute phase protein fibrinogen is upregulated in the lungs of steroid-treated ferrets and SCID mice with PcP [111]. While the lung-specific production of inflammatory mediators may represent the host's unsuccessful attempt to combat *Pneumocystis* infection or to repair lung injury, it may also exacerbate PcP.

In any discussion of inflammatory lung injury, it must be noted that there is a distinction between adaptive (specific) and innate (nonspecific) mechanisms of immune-mediated inflammatory injury. While the available data suggest a direct role for a *Pneumocystis*-specific immune response in injuring the lung, the role of the innate immune system in mediating damage has been more difficult to prove. Polymorphonuclear leukocytes (PMNs) accumulate in the lungs of patients during advanced PcP and have been associated with decreased oxygenation and a poor prognosis [85,89,92,93]. However, whether these cells directly contribute to lung injury or are recruited in response to injury already caused by either *Pneumocystis* or immune-mediated mechanisms is unknown. There have also been several reports of direct stimulation of macrophages by *Pneumocystis* in vitro [103,106,107,109,110]. Whether *Pneumocystis*-stimulated macrophages actually contribute to lung injury or are involved in initiating a specific immune response remains undetermined. Arguing against a contribution of innate mechanisms to inflammatory injury are data obtained from the study of SCID mice, which have a functional innate immune system but severely impaired adaptive immunity. These mice develop large burdens of *Pneumocystis* but show little evidence of inflammation until large organism burdens are achieved and they are near death [17,112,113]. Consequently, the contribution of specific immune-mediated inflammation to lung injury during PcP has received considerably more attention recently. Injurious roles have been suggested for CD4⁺ and CD8⁺ T cells in different patient populations suffering from PcP. The remainder of this chapter focuses on specific immune-mediated mechanisms of lung injury during PcP.

The degree and nature of the immune involvement during PcP may vary in different patient populations. For example, AIDS patients often have a subacute and subtle onset of PcP, while non-AIDS PcP patients present more abruptly with acute disease [12,114,115]. It has also been noted that AIDS patients typically harbor higher organism burdens but demonstrate less pulmonary inflammation, lower rates of admission to intensive care units, less use of mechanical ventilation, and lower mortality than non-AIDS patients with PcP [116,117]. In contrast, AIDS patients who demonstrate a rapid recovery of CD4⁺ T lymphocytes after

institution of combined antiretroviral therapy develop rapid pulmonary decompensation in response to preexisting pulmonary infections, including *Pneumocystis*. This clinical syndrome has been termed “immunorestitution disease” [118,119]. Thus, variation in immunocompetency among AIDS patients may affect their ability to mount an immune-mediated inflammatory response to *Pneumocystis* and may account for variability in the severity of PcP from one AIDS patient to the next. Elucidating the relative contributions of the immune response and the organism burden during different clinical manifestations of PcP is critical to identifying the optimal effective therapeutic combination for these patient populations.

Animal models have been invaluable to the study of mechanisms of lung injury related to PcP. In order to closely mimic the pathological characteristics of PcP in the various clinical settings in which it is manifest, several different animal models have been developed. Chronically corticosteroid-treated rats and ferrets and SCID mice are severely immunosuppressed and extremely sensitive to *Pneumocystis* infection. Therefore they can develop large *Pneumocystis* burdens with little evidence of immune-mediated injury. However, addition of *Pneumocystis* sensitized CD4⁺ T lymphocytes to infected corticosteroid-treated rats [120] or immune reconstitution of infected SCID mice can mimic the CD4⁺ T cell-mediated injury characteristic of immunorestitution-associated PcP in humans [16,113,121]. In these models, lung injury is dependent on the organism burden and the degree of CD4⁺ T-cell recovery. In contrast, AIDS patients typically develop *Pneumocystis* infections when CD4⁺ T-cell numbers drop. In this patient population, CD8⁺ T cells accumulate in the lung and are thought to contribute to lung injury associated with PcP. To study the effects of CD8⁺ T cell-mediated lung injury during AIDS-related PcP, chronically CD4⁺ T cell-depleted mice and, to a lesser extent, SIV-infected macaques have been utilized [113,122]. In both of these models, CD8⁺ T cells accumulate in the lungs in response to *Pneumocystis* infection. Conclusions obtained from these animal models of PcP are discussed in the following sections.

A. CD4⁺ T Lymphocytes and Immunorestitution Disease

As described above, there are several clinical examples that appear consistent with the suggestion that recovery of immune function can result in the exacerbation of the symptoms of PcP. For example, the clinical syndrome of immunorestitution disease-associated PcP has been described in both AIDS and non-AIDS patients. Coincident with recovery of CD4⁺ T lymphocytes associated with successful combined antiretroviral therapy or other clinical conditions, there is a paradoxical worsening of undiagnosed opportunistic infections, including PcP. It has been suggested that the degree of lung damage is a function of the organism burden present at the time of immune recovery and of the extent of that immune recovery

[119]. Thus, the severity of immunorestitution-associated PcP depends on the patient's ability to mount an inflammatory response against the preexisting infection and is likely related to the function of CD4⁺ T lymphocytes.

Several controlled animal studies have modeled immunorestitution disease and directly addressed the contribution of the host's immune response to lung injury during PcP. Roths and Sidman described a potentially lethal, CD4⁺ T cell-dependent hyperinflammatory response following immune reconstitution of *Pneumocystis*-infected SCID mice [121,123]. Although immune recovery allowed the susceptible SCID mice to mount a response against *Pneumocystis*, the CD4⁺ T cell-mediated collateral injury produced by that response was severe. Similar results were reported using the steroid-treated rat model of PcP. When *Pneumocystis*-sensitized CD4⁺ T lymphocytes are transferred to rats with preexisting *Pneumocystis* infection, a pulmonary hyperinflammatory response is also observed and is associated with significant mortality [120]. A recent study has reported that CD4⁺ T lymphocytes expressing the CD25⁻ phenotype are responsible for the hyperinflammatory response observed in reconstituted SCID mice, and that CD25⁺ CD4⁺ T cells can suppress this response [124].

Subsequent experiments using immunologically reconstituted and nonreconstituted *Pneumocystis*-infected SCID mice more precisely defined the specific immune-mediated inflammatory events that lead to pulmonary dysfunction during PcP [17,112]. When *Pneumocystis*-infected SCID mice are reconstituted, they mount an intense inflammatory response against the organism and exhibit decreased lung compliance and arterial oxygenation along with increased respiratory rates as compared to similarly infected, nonreconstituted SCID mice [113]. In addition, direct measurement of the surface tension-reducing properties of the cell-free pulmonary lavage fluid indicated that the inflammatory response severely disrupts pulmonary surfactant function [64]. Impaired pulmonary function correlates temporally with increased pulmonary cytokine and chemokine production [17,112,113] and the recruitment of alveolar macrophages, lymphocytes, and neutrophils to the lung. Despite having significant organism burdens, nonreconstituted mice exhibit little notable inflammation, normal pulmonary function, and normal surfactant properties. Although CD4⁺ T cells are necessary for the resolution of PcP [16,125,126], these experiments provided definitive evidence that the inflammatory response, triggered by immune recognition of *Pneumocystis*, can contribute significantly to respiratory impairment and lung injury in certain clinical settings. Figure 1 depicts the temporal correlation of pulmonary inflammation, as represented by TNF- α gene expression, with impaired pulmonary function following immune reconstitution and recovery of infected SCID mice. Further studies are required to determine the contribution of CD4⁺ T cells and/or other specific cell populations and inflammatory mediators to this process. Ideally, one would want to block the injurious inflammatory response while preserving the protective response required for the clearance of *Pneumocystis*.

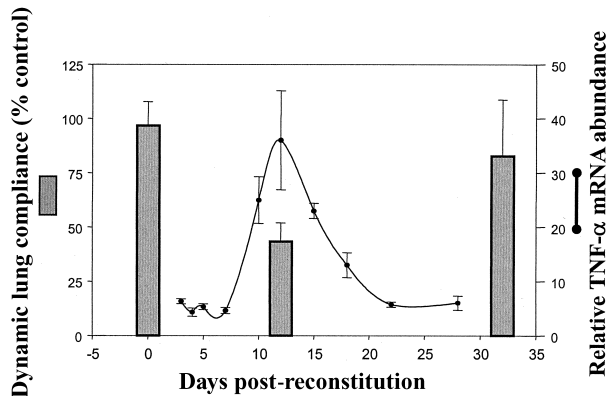


Figure 1 Temporal correlation of decreased specific dynamic lung compliance with pulmonary inflammation following immune reconstitution of *P. carinii*-infected SCID mice. Dynamic lung compliance and pulmonary TNF- α mRNA abundance were measured at various times post-reconstitution. The time course of TNF- α mRNA abundance serves as a reliable marker of pulmonary inflammation in this model. Pulmonary inflammation is minimal and lung compliance normal in infected mice at 0 days post-reconstitution. However, during the peak inflammatory phase following reconstitution (12 days post-reconstitution), lung compliance is dramatically decreased. By 32 days post-reconstitution, the infection has been cleared, pulmonary inflammation is resolving, TNF- α levels have returned to baseline, and lung compliance measurements approach normal values. (From Ref. 113.)

B. CD8⁺ T Lymphocyte-Mediated Lung Injury

AIDS patients are severely immunocompromised and typically diagnosed with higher numbers of *Pneumocystis* while at the same time seeming to display less inflammation and pulmonary compromise than non-AIDS patients [114,116]. It has been suggested that this phenomenon is related to the greater ability of non-AIDS patients to mount a specific immune response against *Pneumocystis*, which can provide some defense against the organism but also has adverse effects on pulmonary function. However, even in AIDS patients with profound reductions in CD4⁺ T cell numbers, it appears that inflammatory mechanisms contribute to lung injury. For example, bronchoalveolar lavage fluid IL-8 and neutrophil concentrations, but not organism numbers, correlate with decreased oxygenation, greater risk of mechanical ventilation and a poorer prognosis in AIDS-related PcP [85,93]. Furthermore, clinical studies of AIDS patients and controlled animal studies have both demonstrated that inflammatory mediators are released and immune and inflammatory cells recruited to the lung in response to *Pneumocystis* even in the absence of fully functional CD4⁺ T cell-mediated immunity

[103,105,106,109,127,129]. In support of a role for specific immunity in lung injury is that *Pneumocystis* infection significantly increases the number of CD8⁺ T lymphocytes in the bronchoalveolar lavage fluid of HIV-infected humans, SIV-infected macaques, steroid-treated rats, and CD4⁺ T cell-depleted mice [88,122,127,128,130,132]. The importance of CD8⁺ T-cell recruitment to both host defense and lung injury has been directly studied in animal models of infection.

While it has been demonstrated that, under certain conditions, the CD8⁺ T-cell population can convey some protective benefit against *Pneumocystis* [128,133,134], only recently has the direct contribution of these cells to lung injury and respiratory impairment been evaluated [64,113]. In the absence of CD4⁺ T cells, *Pneumocystis* infection causes a progressive increase in the number of CD8⁺ T cells recruited to the lung. The infiltrating CD8⁺ T cells are specific for *Pneumocystis* antigens [133] and are associated with decreased lung compliance, impaired oxygenation, increased alveolar-capillary permeability, and pulmonary surfactant dysfunction [64,113]. Furthermore, similar to reports of severe AIDS-related PcP in humans, a dramatic influx of neutrophils into the lung is also characteristic of CD8⁺ T cell-mediated inflammation. In contrast, *Pneumocystis*-

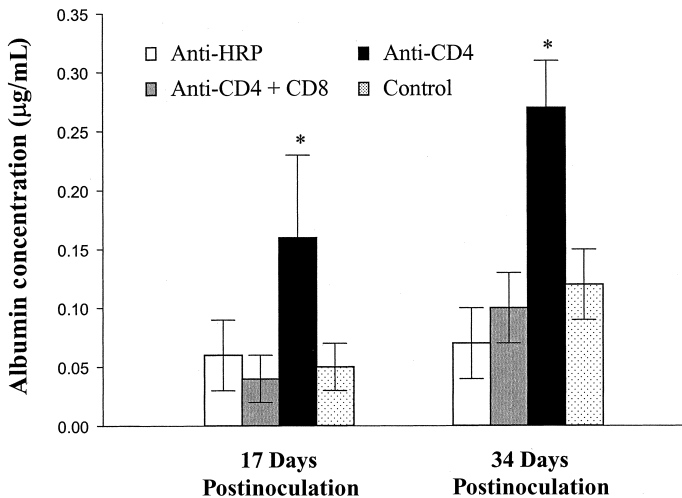


Figure 2 Effect of CD8⁺ T cells on alveolar-capillary permeability during PcP in mice. Albumin content was measured in the bronchoalveolar lavage fluid of CD4-depleted, CD4- and CD8-depleted, and anti-horseradish peroxidase-treated mice inoculated with *P. carinii* 17 or 34 days previously. Controls were uninfected C57BL/6 mice. Values are means $\pm$ 1 SD ($n=5$). * $p<0.05$ compared to CD4- and CD8-depleted, anti-HRP-treated, and uninfected mice at 17 and 34 days postinoculation. (From Ref. 113.)

infected CD4⁺ T cell-depleted mice that are additionally depleted of CD8⁺ T cells exhibit improved pulmonary function in all parameters assessed. The depletion of CD8⁺ T cells resulted in improved lung function, improved surfactant activity, and decreased neutrophil recruitment. Although these mice were significantly improved, they were not totally normal, indicating that *Pneumocystis* itself does cause some lung injury. However, in these studies, the contribution of CD8⁺ T cells was significant. Figure 2 depicts the CD8⁺ T lymphocyte-dependent increase in alveolar permeability present in CD4⁺ T cell-depleted mice with PcP. Thus, in this murine model of PcP, CD8⁺ T cell-mediated inflammation contributes significantly to lung injury and respiratory impairment. In contrast to the clear-cut beneficial effect of CD4⁺ T cells in the control of *Pneumocystis* infection, CD8⁺ T cells induce an ineffective immune response that contributes more to injury than to resolution of disease. Further studies are required to determine the exact roles of CD8⁺ T cells and neutrophils and whether they contribute similarly to lung injury during AIDS-related PcP in humans.

IV. Summary

Lung injury during PcP stems from a combination of many contributing factors derived from both the organism and the host. While there is evidence that *Pneumocystis* and its gene products can injure the lung directly, there is equally compelling evidence supporting a role for the host's immune response in PcP related lung injury. Thus, it is probable that lung injury is due to a combination of these factors, and is dependent on the clinical setting in which PcP is manifest. When PcP develops in patients with chronic, severe immunosuppression, such as those suffering from AIDS, the disease may be characterized by higher numbers of *Pneumocystis* organisms but less inflammation. Therefore direct *Pneumocystis*-mediated mechanisms of lung injury may play a more prominent role than inflammation in this clinical setting. In contrast, when PcP manifests in the setting of immunorestitution disease or in patients who are less immunosuppressed, the immune response to the organism may make the greater contribution to lung damage. While the inflammatory response may help to control organism numbers, it also produces significant collateral damage to the lung. Therefore careful individual consideration must be given to the treatment regimen administered to a specific patient. Ironically, these observations suggest that administration of an anti-inflammatory or immunosuppressive agent to an already immunosuppressed individual may have benefit. Such therapies may improve the clinical symptoms of PcP but may also impair the host's ability to fight *Pneumocystis*. Thus, it is likely that a combination of anti-inflammatory and antimicrobial therapy will be needed for optimal treatment of PcP.

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15

Clinical Presentation and Diagnosis of *Pneumocystis* Pneumonia in HIV-Infected Patients

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I. Introduction

The clinical presentation and diagnosis of *Pneumocystis* pneumonia (PcP) in human immunodeficiency virus (HIV)–infected patients can be challenging for several reasons. First, PcP is the HIV-identifying diagnosis in a significant proportion of patients, and clinicians must maintain a high index of suspicion in evaluating a patient who has no known HIV diagnosis but whose clinical and radiographic presentation is compatible with PcP. Although PcP has a characteristic clinical and radiographic presentation, the extent to which the “classical” presentation is seen depends on several factors, including the use of *Pneumocystis* prophylaxis. Furthermore, the clinical and radiographic presentation of PcP overlaps that of other HIV-associated pulmonary diseases. Occasionally, the chest radiograph—which is the cornerstone of the evaluation of suspected PcP—will be normal, and additional tests, such as chest high-resolution computed tomography (HRCT) or pulmonary function tests (PFTs), may be indicated. PcP–associated acute respiratory failure requiring mechanical ventilation has a significant mortality; prompt diagnosis and initiation of treatment are therefore critical. Finally, there is no universally agreed on diagnostic algorithm for suspected PcP, and institutions report different degrees of success with sputum induction and bronchoscopy with bronchoalveolar lavage (BAL).

This chapter reviews the incidence of PcP both as an AIDS-defining diagnosis as well as in cohorts of HIV-infected patients. It also discusses risk factors for PcP, describes the clinical and radiographic presentation of PcP, the use of tests such as chest HRCT and PFTs in the evaluation of suspected PcP, and outlines the algorithm used at San Francisco General Hospital (SFGH) for the diagnosis of PcP. Treatment and prophylaxis of PcP are discussed in Chapter 20.

II. Incidence

Prior to the HIV/AIDS epidemic, *Pneumocystis pneumonia* was unusual, affecting almost exclusively immunocompromised persons [1]. During a 3-year period from November 1967 [when the Parasitic Disease Drug Service at the Centers for Disease Control (CDC) became the sole supplier of pentamidine isethionate in the United States] to December 1970, 194 U.S. patients were diagnosed with confirmed PcP. In 1981, the description of PcP in 15 previously healthy men who either had sex with other men (MSM) and/or who were injection drug users (IDU) heralded the onset of the HIV/AIDS epidemic [2,3], which currently affects an estimated 42 million people worldwide [4]. Although the incidence of PcP has declined dramatically in the United States, western Europe, and areas of the world where combination antiretroviral therapy is widely available, PcP is increasingly described in Africa, Asia, and Latin America, where greater than 90% of the estimated people living with HIV/AIDS reside and access to combination antiretroviral therapy and even *Pneumocystis* prophylaxis are limited.

A. United States

Throughout the HIV/AIDS epidemic, PcP has been the leading AIDS-defining opportunistic infection in the United States. PcP, either with or without concurrent Kaposi's sarcoma, was the AIDS-defining diagnosis for 580 of the first 1000 AIDS cases [5]. At its peak, there were greater than 20,000 cases of PcP reported to the CDC as AIDS-defining diagnoses each year from 1990 to 1993 [6–9]. Subsequently, the incidence of PcP has undergone a dramatic decline. The use of *Pneumocystis* prophylaxis and combinations of antiretroviral drugs contributed to this decline [10]. In 1997, the last year in which the CDC's annual HIV/AIDS Surveillance Report presented data on specific AIDS-defining diagnoses, the report included fewer than 9500 cases of PcP [11]. Since PcP was never a reportable disease unless it was an AIDS-defining diagnosis, current data on it come from several different cohorts.

PcP is a frequent opportunistic infection in cohorts of HIV-infected patients. In the CDC-led Adult and Adolescent Spectrum of HIV Disease (ASD) cohort, PcP was the most common AIDS-defining opportunistic infection to occur first (36% of the 12,982 persons diagnosed with AIDS) and the most common incident

AIDS-defining opportunistic infection (274 cases per 1000 person-years) from 1992 to 1997 [12]. It was also the most common AIDS-defining opportunistic infection to have occurred during the course of AIDS, and 53% of persons who died with AIDS had PcP diagnosed at some time during their lives. However, the incidence of PcP in the cohort decreased from 108 cases per 1000 person-years in 1992 to 46 cases per 1000 person-years in 1997. A detailed examination of the PcP cases occurring in the ASD cohort from 1996 to 1998 [the period when highly active antiretroviral therapy (HAART) first came into use] found that 46% of PcP cases occurred in persons without prior medical care and without a prior known HIV diagnosis [13]. Among patients with a known HIV diagnosis who were in medical care, one study found that patient nonadherence with prescribed *Pneumocystis* prophylaxis and use of a prophylactic regimen other than trimethoprim-sulfamethoxazole accounted for the majority of the PcP cases seen [14,15]. A study from the CDC-led HIV Outpatient Study (HOPS) found that the incidence of *Pneumocystis* prophylaxis failure was 4.6 PcP cases per 100 person-years on prophylaxis [16]. In a multivariate analysis, the only significant predictors of prophylaxis failure were the use of a prophylactic regimen other than trimethoprim-sulfamethoxazole, a history of prior PcP, and a CD4 lymphocyte cell count of $<50/\text{mm}^3$.

Among children, the National Heart, Lung and Blood Institute Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted HIV Infection (P²C² HIV) Study found that PcP was a frequent cause of pulmonary disease [17]. In this study of 600 infants born to HIV-infected women, 93 were found to be infected with HIV. Among these HIV-infected infants, PcP was more common than both viral and bacterial pneumonia (9.5 PcP cases per 100 child-years compared to 7.1 cases per 100 child-years and 3.6 cases per 100 child-years, respectively).

At San Francisco General Hospital (SFGH), PcP accounted for the majority of pneumonias seen among hospitalized HIV-infected persons until 1995. Throughout the epidemic, we have used a standardized approach to pursue a definitive diagnosis of PcP in virtually all cases [18,19]. At our peak, in 1992, we diagnosed nearly 300 cases of PcP at SFGH (Fig. 1). Subsequently, we saw a decline in the annual number of PcP cases [20], followed by a plateau. Since 1997, we have diagnosed 40 to 85 cases of PcP yearly. In concert with the ASD cohort, one-quarter of the SFGH cases of PcP in the HAART era occurred in persons without a prior diagnosis of HIV and one-half of our cases occurred in persons who, despite knowledge of their HIV infection, were neither on HAART nor on *Pneumocystis* prophylaxis. Only one-quarter of the PcP cases occurred in persons on prophylaxis, with a small subset of these patients also on HAART.

B. Europe

PcP remains the leading AIDS-indicating opportunistic infection in Europe. In contrast to the United States, the 51 countries of the World Health Organization

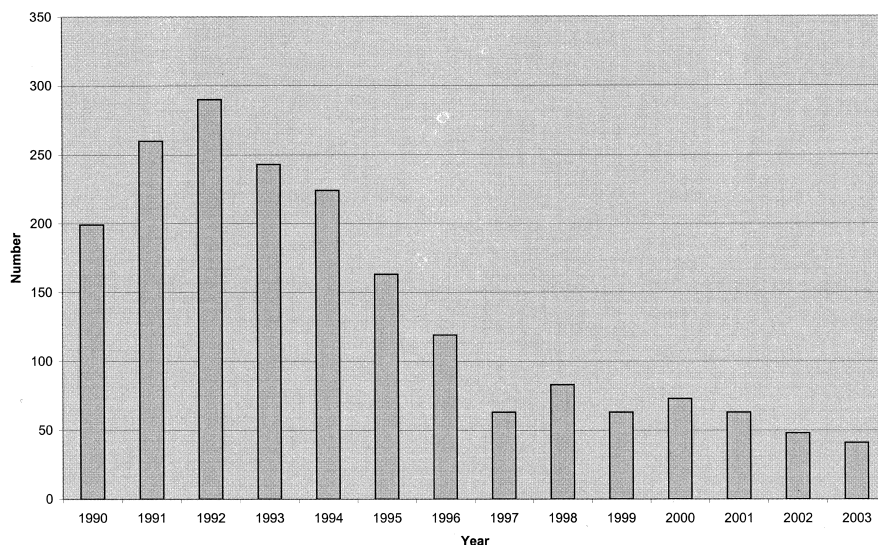


Figure 1 Microscopically confirmed cases of PcP diagnosed at San Francisco General Hospital, 1990–2003.

(WHO) European Region continue to collect and present data on PcP as an AIDS-indicating opportunistic infection. From 1999 to 2001, PcP was the leading AIDS-indicating opportunistic infection, accounting for 22 to 25% of AIDS-indicating diagnoses in adults/adolescents [21–23]. PcP is a frequent opportunistic infection in European cohorts of HIV-infected patients. In the EuroSIDA study, PcP was the most common serious opportunistic infection and occurred in 9% of the AIDS-defining illnesses from 1994 to 1998 [24]. Among AIDS-defining illnesses, this proportion due to PcP remained stable throughout the course of the study, ranging from 10% in 1994 to 11% in 1998. However, in this observational study of over 7300 HIV-infected patients followed in 52 European HIV clinics, the number of subjects who developed PcP in a given year declined from 49 in 1994 to 10 in 1998.

C. Developing Countries

PcP appears to be a frequent opportunistic infection in HIV-infected patients throughout the world. At present, precise data on PcP either as an AIDS-defining diagnosis or in cohorts of HIV-infected persons is limited mostly to single-institution studies. These studies include different study designs, study populations, and criteria for defining PcP (clinical vs. microscopic confirmation) that limit firm conclusions. Nevertheless, in contrast to early reports from both clinical and

autopsy studies [25–29], PcP appears to be a frequent opportunistic infection in HIV-infected patients throughout the world, including Africa [30–33]. A review of PcP in HIV-infected patients in Africa, Asia, and Central and South America demonstrated that PcP comprised a significantly greater proportion of cases of pneumonia than previously reported [34]. Importantly, a substantial proportion of these HIV-infected patients with PcP have been found to have concurrent tuberculosis—a finding that complicates the diagnosis and treatment [35]. As the HIV/AIDS epidemic progresses in these regions, it is increasingly important to develop diagnostic and treatment algorithms that can be used in settings where resources, diagnostic tests, and medications are limited [36].

In Africa, HIV-infected infants ≤ 1 year of age appear to be especially vulnerable to PcP [37–43]. In one clinical study, children 2 to 24 months of age who were either known or suspected of being HIV-infected and who were hospitalized for severe pneumonia underwent testing of sputum and nasopharyngeal aspirate for *Pneumocystis* [39]. Using a direct monoclonal antibody immunofluorescent stain, *Pneumocystis* was identified in 51 out of 105 (49%) children from induced sputum (37 of 105, or 35%) or nasopharyngeal aspirate (26 of 101, or 26%) or both. In another clinical study, *Pneumocystis* was identified in 101 out of 231 (44%) episodes of pneumonia among 185 HIV-1-infected children (median age, 4.5 months) [40]. In autopsy studies, *Pneumocystis* has been found in 29 to 67% of HIV-infected children who died of pneumonia [41–43].

III. Risk Factors for *Pneumocystis* Pneumonia

Several risk factors for PcP have been defined, while other potential factors remain speculative. The U.S. Public Health Service (USPHS) and the Infectious Diseases Society of America (IDSA) guidelines for preventing opportunistic infections recommend that HIV-infected adults or adolescents (including those on HAART) who have CD4 lymphocyte counts $< 200/\text{mm}^3$ or a history of oropharyngeal candidiasis receive primary *Pneumocystis* prophylaxis, and persons with prior PcP receive secondary prophylaxis [44]. HIV-infected adults or adolescents without these criteria but who have a CD4 lymphocyte percentage $< 14\%$ or a prior AIDS-defining illness should be considered for *Pneumocystis* prophylaxis. These recommendations also apply to HIV-infected women who are pregnant. The guidelines do acknowledge that consideration for temporarily discontinuing prophylaxis during the first trimester (because of concerns regarding possible teratogenicity of the oral prophylaxis regimens) or for using aerosolized pentamidine (because of lack of systemic absorption) is reasonable. Once started, HIV-infected persons should remain on prophylaxis for life unless their CD4 lymphocyte counts increase from < 200 to $> 200/\text{mm}^3$ for at least 3 months as a result of HAART.

Infants born to HIV-infected mothers should receive *Pneumocystis* prophylaxis at 4 to 6 weeks of age, until the infant's HIV serostatus can be conclusively determined [44]. Those infants who are subsequently determined to be uninfected with HIV can discontinue prophylaxis. HIV-infected infants and infants whose HIV serostatus remains unknown should continue to receive prophylaxis for the first year of life. After the first year, the need for subsequent prophylaxis is based on age-specific CD4 lymphocyte count thresholds: for children 1 to 5 years of age, a CD4 lymphocyte count $<500/\text{mm}^3$ or CD4 lymphocyte percentage $<15\%$; children ≥ 6 years of age, a CD4 lymphocyte count $<200/\text{mm}^3$ (as for adults and adolescents) or CD4 lymphocyte percentage $<15\%$.

These recommendations are based on several landmark studies, some of which are highlighted below.

A. CD4 Lymphocyte Count

The CD4 lymphocyte count is a strong predictor of an HIV-infected patient's risk of developing PcP. PcP was perhaps the first disease in which documentation of the CD4 lymphocyte count was used to assess the risk for the development of opportunistic infection. Early in the epidemic, the CDC and colleagues in New York, San Francisco, Los Angeles, and Atlanta conducted a national case-control study of PcP and Kaposi's sarcoma in homosexual men [45,46]. These patients were found to have lymphopenia, specifically a deficiency in the T-helper cells, which resulted in a reversal of the ratio of T-helper to T-suppressor cells. Masur and colleagues, in a review of episodes of opportunistic pneumonia diagnosed at the Clinical Center of the National Institutes of Health (NIH), found that 46 of 49 patients (94%) diagnosed with PcP had CD4 lymphocyte counts $\leq 200/\text{mm}^3$ and 47 patients (96%) had CD4 lymphocyte percentages $\leq 20\%$ [47]. The Multicenter AIDS Cohort Study (MACS) demonstrated a significantly increased risk for PcP among HIV-infected participants with CD4 lymphocyte counts $\leq 200/\text{mm}^3$ at study entry [48]. This study examined 1665 HIV-infected MSM without a prior AIDS diagnosis and without use of *Pneumocystis* prophylaxis. Subjects with CD4 lymphocyte counts $\leq 200/\text{mm}^3$ at study entry had a nearly fivefold greater risk of developing PcP than did subjects who had CD4 lymphocyte counts $>200/\text{mm}^3$ (RR = 4.9, 95% CI = 3.1–8.0). Among subjects with baseline CD4 lymphocyte counts $\leq 200/\text{mm}^3$, greater than 18% developed PcP within 1 year and one-third developed PcP within 3 years. In this study, the presence of fever ≥ 2 weeks and oral thrush ≥ 2 weeks also independently influenced the risk of progression to PcP (RR = 2.15, 95% CI = 1.02–4.54; RR = 1.86, 95% CI = 1.13–3.06, respectively). The results of this study led directly to the publication of the initial CDC recommendations for the use of prophylaxis against *Pneumocystis* in HIV-infected persons [49]. Stansell and colleagues in the Pulmonary Complications of HIV Infection Study (PCHIS) con-

firmed the importance of the CD4 lymphocyte count as a risk factor for PcP and extended these findings to a broader patient population [50]. The PCHIS was a prospective multicenter cohort study of pulmonary disease in HIV-infected subjects. The PCHIS cohort included 1184 HIV-infected subjects and approximated 1990 U.S. AIDS cases with regard to gender, race, and ethnic background as well as HIV transmission category. Thus, the PCHIS differed from the earlier studies in that it included men and women IDUs and heterosexual women of all races and ethnic backgrounds. This report also included patients who were receiving *Pneumocystis* prophylaxis. Overall, 95% of the 136 first-episode PcP cases occurred in subjects with CD4 lymphocyte counts $\leq 200/\text{mm}^3$, and 79% had CD4 lymphocyte counts $\leq 100/\text{mm}^3$. The median CD4 lymphocyte count at the time of PcP diagnosis was $29/\text{mm}^3$. The rate of PcP among persons with baseline CD4 lymphocyte counts $\leq 200/\text{mm}^3$ was 8.08 cases per 100 person-years and was higher (11.13 cases per 100 person-years) for those with baseline CD4 lymphocyte counts $\leq 100/\text{mm}^3$ than for persons with baseline CD4 lymphocyte counts of 101 to $200/\text{mm}^3$ (5.95 cases per 100 person-years). In a multivariate analysis, the risk for PcP was associated with the CD4 lymphocyte count, *Pneumocystis* prophylaxis, and, intriguingly, race. In the authors' analysis, black subjects were significantly less likely to develop PcP than nonblacks. Finally, Kaplan and the ASD study examined 2625 initial PcP episodes in 22,698 HIV-infected subjects [51]. Cox proportional hazard analysis revealed that decreasing CD4 lymphocyte count, prior AIDS-defining illness, prior non-*Pneumocystis* pneumonia, oral thrush, and unexplained fever for ≥ 2 days were all risk factors for PcP, while the use of *Pneumocystis* prophylaxis was strongly protective against PcP.

In HIV-infected children, the incidence of PcP is related to age. Most cases of PcP among them occur between 3 and 6 months of age [52]. At this age, many infants have yet to be diagnosed with HIV infection, and these infants frequently develop PcP at CD4 lymphocyte counts $>200/\text{mm}^3$ [53]. In a CDC-led surveillance study of U.S. children who had acquired HIV infection perinatally, PcP was reported in 1374 of 3665 (37%) perinatally acquired cases [54]. Over half of these cases occurred in infants between 3 and 6 months of age. In a review of 300 HIV-infected children diagnosed with PcP, 199 (66%) had never received *Pneumocystis* prophylaxis [55]. Exposure to HIV was first identified ≤ 30 days before the diagnosis of PcP for 118 of these 199 (59%) children. Among the 129 infants <1 year of age, the CD4 lymphocyte count declined by almost $1000/\text{mm}^3$ during the 3 months before the diagnosis of PcP. Among infants in whom a CD4 lymphocyte count was determined within 1 month of the diagnosis of PcP, 18% (20 of 113) had a CD4 lymphocyte count of at least $1500/\text{mm}^3$, a finding that led to a change in the guidelines for infants <1 year of age [56].

In patients who experience a dramatic rise in their CD4 lymphocyte counts due to HAART, this count remains a strong predictor of an HIV-infected patient's risk of PcP. Several studies have demonstrated that the risk of PcP is low in

HIV-infected adult and adolescent patients who had CD4 lymphocyte counts $<200/\text{mm}^3$ but whose most recent CD4 lymphocyte counts have been $>200/\text{mm}^3$ for at least 3 months as a result of HAART [57–68]. Presumably, the risk of PcP in HIV-infected children who had a similar increase in their CD4 lymphocyte count as a result of HAART will also be low, although data are only now being reported [44,69,70]. Therefore, in patients who are responding to HAART with a rise in CD4 lymphocyte counts from <200 to $>200/\text{mm}^3$, the most recent (rather than the nadir) CD4 lymphocyte count is the best indicator of the risk for PcP.

B. HIV-1 RNA Level (Viral Load)

The HIV-1 RNA level is an independent predictor of the risk for progression to AIDS and death [71,72]. Several studies have also shown that the HIV-1 RNA level is an independent predictor for the development of opportunistic infections, including PcP [73–76]. Lyles and colleagues in the MACS cohort found that for any baseline CD4 lymphocyte count range ($<200/\text{mm}^3$, 200 to $349/\text{mm}^3$, and $\geq 350/\text{mm}^3$) the risk for PcP increased with increasing baseline HIV-1 RNA levels ($<30,000$ copies per milliliter, $30,000$ to $75,000$ copies per milliliter, and $\geq 75,000$ copies per milliliter) [73]. Similarly, Kaplan and colleagues in the ASD study found that, although a decreasing CD4 lymphocyte count was the strongest predictor for risk of opportunistic infection, an increasing HIV-1 RNA level was independently associated with an increased risk [76]. While the HIV-1 RNA level has been demonstrated to be a risk factor for PcP that is independent of the CD4 lymphocyte count, clinical guidelines for its use in assessing a patient's risk for PcP have yet to be developed.

C. Natural Reservoir and Person-to-Person Transmission

Potentially, the risk of PcP is associated with exposure to a source of human *Pneumocystis*. However, the natural reservoir for human *Pneumocystis* remains unknown. Both environmental and human reservoirs have been suggested. Gardening and camping or hiking within the preceding 6 months were both independent risk factors associated with an increased risk of PcP in a case-control study of risk factors for pneumonia in HIV-infected persons [77]. Geography has been shown to be a risk factor for PcP, with northern countries and cities typically having a greater risk than southern ones [78–80]. In addition, geography *within* cities has also been shown to be a risk factor for PcP, with specific geographies as defined by zip codes associated with the risk for PcP [81–83]. Finally, as discussed in greater detail in Chapter 19, geography is associated with the risk for specific *Pneumocystis* molecular genotypes [84–86]. Thus, the risk of PcP may be related to exposure to a source of human *Pneumocystis* that is broadly characterized by geography. The identification of the precise reservoir may be

an important step toward prevention, especially in persons who are receiving neither HAART nor *Pneumocystis* prophylaxis.

Reports have suggested that *Pneumocystis* may be transmitted from person to person and that an immunocompromised person exposed to an individual with PcP may be at risk of PcP from that exposure. The general inability to culture human *Pneumocystis* has limited our understanding of its life cycle and its epidemiology and transmission. Animal-to-animal transmission of *Pneumocystis* has been conclusively demonstrated in same-species laboratory animals (see Chap. 6). Presumably a similar model exists for human disease, although the current data in humans are observational and circumstantial. According to traditional theory, PcP in humans results from reactivation of a latent infection that is acquired early in childhood [87–90]. According to this theory, there is little reason for implementing measures designed to avoid or minimize exposure to *Pneumocystis* in adulthood, as such exposure will undoubtedly already have occurred. However, evidence suggests that PcP in humans may also result from a recent exposure to *Pneumocystis*, with a resultant infection. Numerous reports of cluster outbreaks of PcP among different immunocompromised populations support the theory that PcP can result from person-to-person transmission and a recent exposure in the setting of immunosuppression. According to this theory, those infected with HIV and other immunocompromised persons who are at risk for PcP should avoid close contact with any individuals who have PcP.

While data suggest that PcP in humans may result from both reactivation of a latent infection and from a recent infection, it remains unclear which mechanism predominates and under what conditions that mechanism may become active [90]. Therefore the USPHS/IDSA guidelines for preventing opportunistic infections state the following: “Although certain authorities might recommend that HIV-infected persons who are at risk for PcP avoid sharing a hospital room with a patient who has PcP, data are insufficient to support this recommendation as standard practice” [44].

IV. Clinical Presentation

The clinical presentation of PcP in HIV-infected persons differs from the presentation in other immunocompromised persons (Chap. 15). In general, HIV-infected persons present with a subacute onset and a longer symptom duration than other immunocompromised persons [91]. Reports comparing the clinical presentation of PcP in HIV-infected and non-HIV patients have found that HIV-infected patients present with a higher arterial oxygen tension and a lower alveolar-arterial oxygen gradient [91–93] and contain significantly greater numbers of *Pneumocystis* and also significantly fewer neutrophils recovered on BAL than non-HIV patients [92].

A. Symptoms and Signs

Classically, PcP presents with fever, cough, and dyspnea on exertion in both adults and children [94–97]. The cough is usually dry and nonproductive unless a concurrent bacterial infection is present. The cough may be exacerbated by deep breathing and accompanied by “chest tightness,” which results in a short, rapid breathing pattern. Initially, the dyspnea is mild (and therefore frequently dismissed by patients); it usually progresses gradually. Fatigue is a frequent complaint, whereas chest pain (unless due to a pneumothorax), chills, and night sweats are less frequent. Symptoms are often subtle at the onset but are gradually progressive and may be present for weeks and occasionally months before diagnosis. In my experience, patients presenting with PcP as the HIV-identifying diagnosis more often present with weeks to months of symptoms, while those with prior PcP recognize the onset of their characteristic prior symptoms and present earlier. While patients without a prior HIV diagnosis often delay seeking medical attention for a number of reasons (i.e., homelessness, substance use, mental illness, denial), clinicians treating these patients occasionally fail to recognize that the presentation may be due to PcP. Instead, these patients are diagnosed as having a viral illness and/or are treated for bacterial bronchitis or pneumonia (often with multiple courses of different antibiotics) before the specter of HIV infection is raised and the diagnosis of PcP established.

Physical examination is nonspecific. Often vital signs may reveal a temperature $>38.5^{\circ}\text{C}$, tachypnea, and a decreased oxygen saturation. The pulmonary examination is often unremarkable, even in the presence of significant disease and hypoxemia. In one study, the lung examination was noted to be normal in 78 of the 145 (54%) patients with PcP [98]. One maneuver that has been reported to be sensitive for PcP is the elicitation of a cough after deep inspiration. Bilateral fine inspiratory crackles are the most frequent abnormal finding on auscultation [98]. Wheezing suggestive of asthma or airway hyperresponsiveness has been described in patients with PcP [99]. The findings of lung consolidation suggest either severe PcP or a concurrent opportunistic infection, such as bacterial pneumonia. Finally, absent breath sounds indicate the presence of pneumothorax, which requires prompt evaluation (chest radiograph) and management.

B. Laboratory Data

No current laboratory test is specific for PcP [94]. The serum lactate dehydrogenase (LDH) is usually elevated in patients with PcP. Published studies report the sensitivity of an elevated serum LDH for PcP to range from 82 to 100% [98,100–106]. However, a number of caveats accompany the use of the serum LDH as a diagnostic tool for PcP. First, the serum LDH is nonspecific for PcP, and elevations can result from many pulmonary and nonpulmonary etiologies. Thus, an elevated serum LDH cannot be interpreted as diagnostic for PcP. Next,

patients with PcP may have a normal or minimally elevated serum LDH; therefore, a normal serum LDH cannot exclude the diagnosis of PcP. Most of the published studies have been comprised of hospitalized patients with PcP, some of whom had respiratory failure and were in the intensive care units (ICUs). The study that comprised outpatients presenting to an urgent care clinic reported a low sensitivity (83%) of an elevated serum LDH for PcP [103]. One study of patients who developed PcP despite receiving aerosolized pentamidine prophylaxis also reported a low sensitivity (82%) of an elevated serum LDH for PcP [106]. These results suggest that the patient population studied, the severity of PcP, and the use of *Pneumocystis* prophylaxis may all affect the sensitivity of an elevated serum LDH for PcP.

Despite these diagnostic limitations, the serum LDH can be used as a prognostic tool and as a tool to assess response to PcP therapy. Multiple studies have shown a strong correlation between the degree of serum LDH elevation and survival [98,100,102,107]. A high initial serum LDH value or a rising value while a patient is on PcP treatment correlates with a worse prognosis, the failure of therapy, and increased mortality, whereas a low initial value or a declining value on PcP treatment correlates with a better prognosis, a response to therapy, and decreased mortality. Thus, I will never use the serum LDH to decide whether to pursue a diagnosis of PcP (or to treat empirically). However, I obtain a baseline serum LDH in all patients with PcP and will use that value to assist in discussions regarding prognosis and to assess response to PcP therapy.

The arterial blood gas (ABG) is an essential laboratory test in patients with PcP. Typically, patients with PcP will have a decreased arterial oxygen tension, an increased alveolar-arterial oxygen gradient, and a respiratory alkalosis. The ABG should be used to assist in the decisions whether to admit a patient to the hospital, whether adjunctive corticosteroids are indicated ($\text{PaO}_2 \leq 70$ mmHg OR an alveolar-arterial oxygen gradient ≥ 35 mmHg), and as a tool to assess response to PcP therapy. Like the serum LDH, the alveolar-arterial oxygen gradient will decrease toward normal in patients who respond to PcP treatment [102].

C. Chest Radiograph

The chest radiograph is the cornerstone of the evaluation of an HIV-infected patient with suspected PcP. Classically, PcP presents with bilateral, diffuse, symmetrical reticular (interstitial) or granular opacities (Fig. 2) [108–114]. Typically, PcP begins with central or perihilar opacities and a middle-lower lung zone predominance. As with the classical radiographic presentation, these opacities are bilateral and symmetrical and can progress to diffuse involvement if undiagnosed and untreated. However, PcP can also present with unilateral, focal, or asymmetrical opacities (Fig. 3). In my experience, the specific pattern seen is more important than the exact distribution. PcP must be considered in any HIV-infected patient



Figure 2 Chest radiograph of an HIV-infected patient with PcP, revealing the characteristic bilateral diffuse granular opacities. (Courtesy of L. Huang, used with permission.)



Figure 3 Chest radiograph of an HIV-infected patient with PcP presenting with unilateral (left lung) granular opacities. Although PcP usually presents with bilateral, symmetrical opacities, the opacities can be unilateral or asymmetrical; the diagnosis of PcP should still be considered in these cases. (Courtesy of L. Huang, used with permission.)

who is at risk for PcP, has a compatible clinical presentation, and presents with reticular or granular opacities on chest radiograph, regardless of whether the findings are unilateral or bilateral, focal or diffuse, asymmetrical or symmetrical. Clearly though, the presence of bilateral, diffuse, and symmetrical reticular (interstitial) or granular opacities increases the probability of PcP significantly. Huang and colleagues in the PCHIS reported on 467 consecutive HIV-infected patients with new or worsening fever, cough, and/or dyspnea who had a chest radiograph and who were evaluated using a standardized diagnostic algorithm [19]. In this study, patients who had interstitial infiltrates noted on their radiograph were 4.4 times more likely to have PcP than those without this pattern and patients who had interstitial infiltrates that involved five or six of the six defined lung zones (upper, middle, and lower lung zones on the right and left lungs) were 5.3 times more likely to have PcP.

Patients with severe PcP and respiratory failure will invariably have bilateral areas of consolidation. Thin-walled cysts or pneumatoceles are reported in approximately 5 to 34% of PcP cases (Figs. 4 and 5) [111,115–119]. Pneumato-



Figure 4 Chest radiograph of an HIV-infected patient with PcP presenting with characteristic opacities and multiple thin-walled cysts (pneumatoceles). (Courtesy of L. Huang, used with permission.)



Figure 5 Chest radiograph of an HIV-infected patient with PCP, revealing the characteristic reticular-granular opacities as well as three thin-walled cysts (pneumatoceles), indicated by the arrows. (Courtesy of L. Huang, used with permission.)

celes may be present at the time of diagnosis, may develop while a patient is on therapy, and may persist despite successful therapy. The pneumatoceles may be single or multiple in number, small or large in size. Usually pneumatoceles are multiple in number and located in the upper lobes. Importantly, the presence of pneumatoceles predisposes patients to the development of pneumothorax (Figs. 6 and 7). However, pneumothorax may also occur spontaneously in the absence of pneumatoceles demonstrable on chest radiography. In either case, the management of pneumothorax in the setting of PCP is a significant challenge.

Less commonly, PCP may present with a lobar or segmental consolidation, nodules of varying size with or without cavitation [120–122], or a miliary pattern [117]. PCP may also present with granulomatous lesions and with a histological pattern consistent with bronchiolitis obliterans organizing pneumonia (BOOP) [123–126]. Apical or upper-lung-zone disease resembling tuberculosis has been associated with aerosolized pentamidine prophylaxis [127], although this presen-

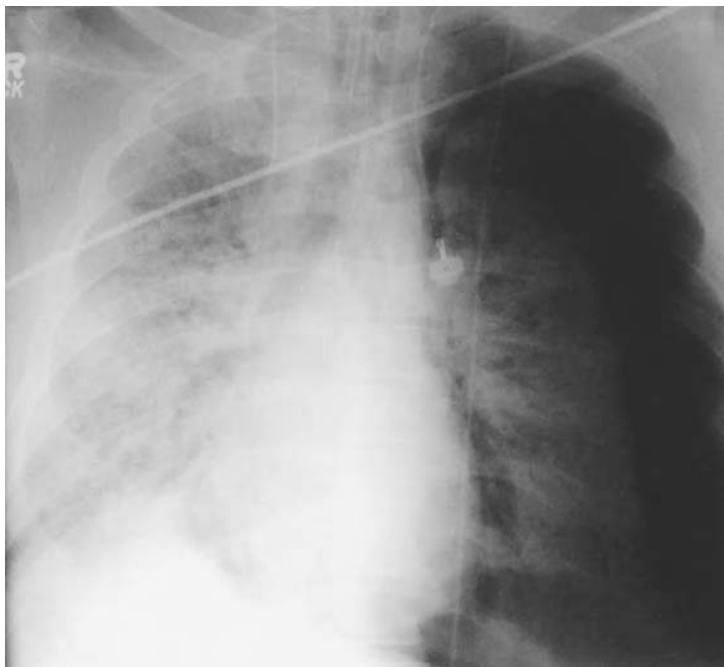


Figure 6 Chest radiograph of an HIV-infected patient with PcP complicated by a left-sided tension pneumothorax. (Courtesy of L. Huang, used with permission.)

tation can also occur in patients receiving other forms of *Pneumocystis* prophylaxis or no preventive therapy (Fig. 8). While reported, intrathoracic adenopathy and pleural effusions are rarely due to PcP [128]. The presence of these radiographic findings should prompt a search for an alternative or at least a coexisting process, such as bacterial pneumonia, tuberculosis, fungal pneumonia, or pulmonary Kaposi's sarcoma.

PcP may present with a normal chest radiograph. Published studies report the incidence of this to be in the range of 0 to 39% [108–114,129]. In the SFGH experience, PcP presents with a normal chest radiograph in fewer than 10% of cases. While patients with PcP and a normal chest radiograph have a better prognosis than those with the classical bilateral diffuse opacities, they often represent a diagnostic challenge and require a timely evaluation before their disease progresses.

There are a few studies on the course of respiratory symptoms and chest radiographic abnormalities in patients successfully treated for PcP [111,130].



Figure 7 Chest radiograph of a critically ill HIV-infected patient with PcP complicated by bilateral pneumothoraces, which required bilateral chest tube placement. Note small residual left-sided pneumothorax. (Courtesy of Chin-Tang Huang, MD, used with permission.)

Patients with PcP often experience a clinical and radiographic worsening in the first 3 to 5 days of PcP therapy. In one study of 104 patients with PcP, 46% of the patients demonstrated a deterioration in the chest radiograph at 1 week [111]. In this same study, approximately one-third of the patients showed no change in their radiographs over the first 3 weeks. In general, the more severe the PcP, the more prolonged the time to clinical and chest radiographic resolution. In practice, I often ask patients to recall the number of days or weeks that they were symptomatic prior to the initiation of PcP therapy and then advise them to expect approximately the same number of days or weeks until symptom resolution.

D. High-Resolution Computed Tomography (HRCT) of the Chest

As described, patients with PcP will have a normal chest radiograph in up to 39% of reported series. Often, these cases can be challenging for the clinician,



Figure 8 Chest radiograph of an HIV-infected patient with PcP presenting with bilateral but asymmetrical (left lung greater than right lung) upper-lobe opacities. (Courtesy of Chin-Tang Huang, MD, used with permission.)

as the vast majority of patients with normal radiographs will not have PcP, and the decision to pursue a diagnosis of PcP relies on both clinical judgment and additional testing. High-resolution computed tomography (HRCT) of the chest is an important test to consider in an HIV-infected patient with a clinical suspicion for PcP but with a normal chest radiograph. Classically, PcP presents with bilateral ground-glass opacities on HRCT (Fig. 9) [131–138]. These opacities may be accompanied by multiple pneumatoceles (Fig. 10). Although the presence of ground-glass opacities on chest HRCT is nonspecific and may be seen in a number of pulmonary disorders, its absence argues strongly against the presence of PcP. In one study, all six HIV-infected patients with PcP who had normal chest radiographs had ground-glass opacities on chest HRCT [137]. More importantly, none of the 40 patients with clinically suspected PcP, a normal or nonspecific chest radiograph, and a chest HRCT without ground-glass opacities had PcP on bronchoscopy with BAL or during 60 days of clinical follow-up. In my experience

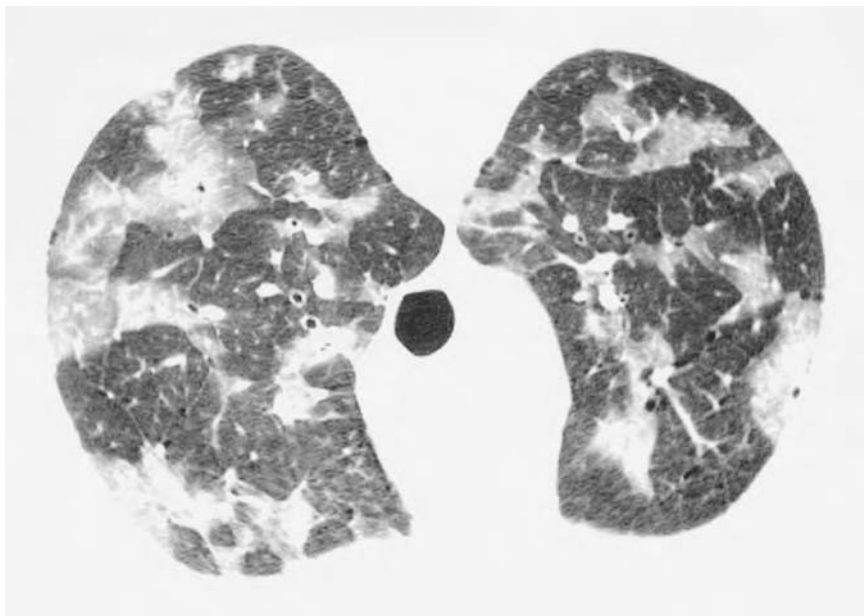


Figure 9 Chest high-resolution computed tomography (HRCT) scan of an HIV-infected patient with PcP who had a normal chest radiograph. HRCT scan demonstrates the characteristic ground-glass opacities. (Courtesy of L. Huang, used with permission.)

at SFGH, we have never diagnosed PcP in an HIV-infected patient who had both a normal chest radiograph and a chest HRCT without ground-glass opacities.

E. Pulmonary Function Tests (PFTs)

Pulmonary function testing, specifically measurement of the diffusing capacity for carbon monoxide (DL_{co}), is another important test to consider in an HIV-infected patient with a clinical suspicion for PcP but with a normal chest radiograph. On pulmonary function testing, patients with PcP often demonstrate a restrictive ventilatory defect with decreased lung volumes and increased airflows. However, a more sensitive indicator of alveolar capillary block, and hence PcP, is a decrease in the DL_{co} [139]. Typically, patients with PcP will have a decreased DL_{co} . Published studies report the sensitivity of a decreased DL_{co} for PcP to range from 89 to 100% [19,109,140–145]. Although the presence of a decreased DL_{co} is nonspecific and may be seen in a number of pulmonary disorders, a normal DL_{co} argues strongly against the presence of PcP. In one study, only 2 of the 103 consecutive HIV-infected patients with clinically suspected PcP who

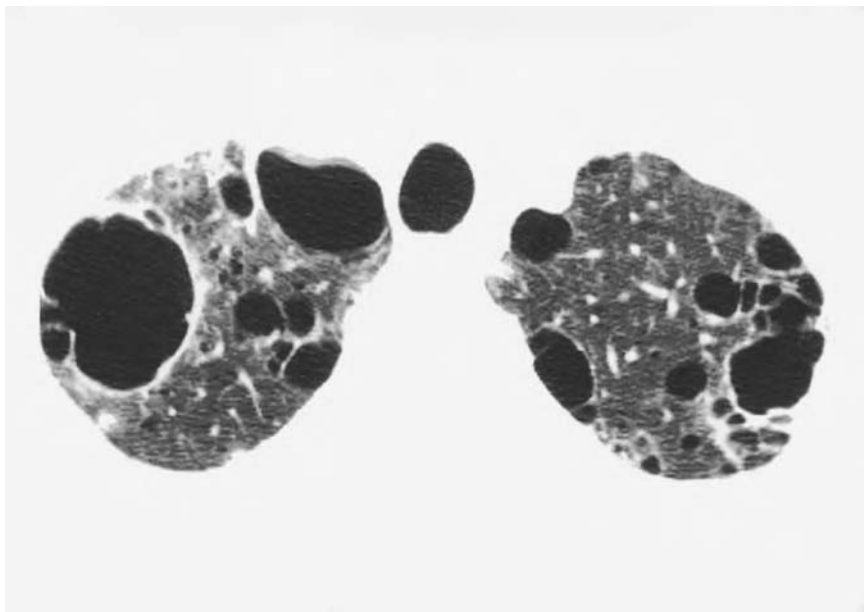


Figure 10 Chest high-resolution computed tomography (HRCT) scan of the HIV-infected patient with PcP whose chest radiograph is depicted in Fig. 5. HRCT scan demonstrates multiple thin-walled cysts (pneumatocoeles). (Courtesy of L. Huang, used with permission.)

had both a normal or unchanged chest radiograph and a $DL_{co} > 75\%$ predicted had PcP diagnosed [19].

F. Other Tests

Several other tests have been reported to be useful in the diagnostic approach to an HIV-infected patient with suspected PcP. Non-invasive exercise oximetry testing may be a useful screening test in an outpatient setting [146–148]. Patients with PcP will often have a decline in their oxygen saturation with exertion. Invasive exercise arterial oxygen testing has also been used [149]. This study showed that a normal exercise arterial oxygen test ruled out the diagnosis of PcP. Gallium-67 scanning has been the most frequently used nuclear medicine study in the evaluation of suspected PcP. Like the studies discussed above, gallium scans are sensitive but nonspecific indicators of PcP [150–155]. This also appears to be the case with other nuclear medicine studies [156–158].

G. Effect of Prophylaxis on Presentation

Studies suggest that the use of *Pneumocystis* prophylaxis will affect the presentation of PcP. Although trimethoprim-sulfamethoxazole is recommended as the first-line *Pneumocystis* prophylactic regimen, there is limited information on the effect of trimethoprim-sulfamethoxazole prophylaxis on the presentation of PcP [159]. One study of 114 consecutive HIV-infected patients diagnosed with PcP as the sole cause of respiratory symptoms found that patients receiving *Pneumocystis* prophylaxis (predominantly with either dapsone or trimethoprim-sulfamethoxazole) were significantly less likely to present with diffuse chest radiographic disease than patients who were on no prophylaxis (25 vs. 47%, $p < 0.02$) [160]. A prospective randomized study comparing dapsone and aerosolized pentamidine for prophylaxis of *Pneumocystis* found that the 15 patients who developed PcP in each group had similar clinical presentations (initial mean temperature, respiratory rate, chest examination, serum LDH, room air arterial oxygen tension) and chest radiographic patterns [161]. In contrast, several reports have described the effect of aerosolized pentamidine prophylaxis on the presentation of PcP [106,117,127,162–164]. These studies have found that patients who were receiving aerosolized pentamidine for *Pneumocystis* prophylaxis were more likely to present with PcP that had an upper lobe predominance and/or pneumatoceles on chest radiograph compared to patients on no prophylaxis [117,127,162,164].

H. Extrapulmonary Disease

Although extrapulmonary pneumocystosis has been described, possibly in association with the use of aerosolized pentamidine for prophylaxis, most antemortem cases of *Pneumocystis* infection involve only the lungs. Extrapulmonary pneumocystosis has been estimated to occur in less than 1 to 3% of PcP cases [165–169]. Extrapulmonary spread of *Pneumocystis* infection occurs via both lymphatic and hematogenous routes [166,167]. *Pneumocystis* involvement has been described in virtually every tissue and organ [165–170]. Most commonly, the lymph nodes, spleen, liver, and bone marrow are involved. The sole involvement of the ear or choroid of the eye has been associated with an improved outcome. HIV-infected patients with extrapulmonary pneumocystosis may present without concurrent PcP, which complicates its diagnosis [167,169,170]. Often, patients with extrapulmonary disease are asymptomatic and are diagnosed with pneumocystosis at postmortem. The signs and symptoms of extrapulmonary pneumocystosis are nonspecific; when present, however, they are usually referable to the specific tissues or organs involved. Extrapulmonary pneumocystosis can be diagnosed by the examination of tissue biopsies from the affected sites using standard histological techniques. There are no clear guidelines for the treatment of isolated extrapul-

monary disease; presumably patients with concurrent PcP should be treated with the standard 21-day course of therapy.

V. Complications

A. Respiratory Failure

Acute respiratory failure that requires mechanical ventilation remains a common complication of PcP and is associated with a high mortality. In studies of the critical care of HIV-infected patients, respiratory failure accounts for nearly 40 to 50% of ICU admissions, and PcP remains the most frequent cause of respiratory failure [171–177]. Earlier in the HIV/AIDS epidemic, several ICU series of HIV-infected patients found that PcP accounted for 55 to 90% of cases with acute respiratory failure. Subsequent ICU series have noted that PcP accounted for a lower albeit still substantial proportion, in the range of 25 to 50%. Although the overall incidence of PcP at SFGH has declined in the HAART era, greater than one-quarter of the HIV-infected patients admitted to the ICU with acute respiratory failure are admitted due to PcP [177]. In addition, the proportion of patients diagnosed with PcP who require critical care has remained constant in the HAART era [178]. Since 1996, approximately 10 to 15% of the patients diagnosed with PcP each year are admitted to the ICU for management.

The optimal management of acute respiratory failure due to PcP relies on several factors. First, in my opinion, patients must have a confirmed diagnosis of PcP. A number of HIV-associated opportunistic infections can have a clinical and radiographic presentation that is indistinguishable from PcP, and the use of empirical treatment in such critically ill patients is rarely justified. In addition, patients with confirmed PcP should also have an extensive investigation for concurrent opportunistic infections, especially pulmonary infections that affect mortality. HIV-infected patients repeatedly fail to conform to “Occam’s razor” (suggesting that the simplest theory be given priority) and bacterial or mycobacterial infections and pulmonary Kaposi’s sarcoma often coexist with PcP [179–181]. *Cryptococcus neoformans* and cytomegalovirus pneumonias often present with identical clinical and radiographic presentations to PcP; when concurrent with PcP, they are difficult to detect unless specifically sought. As discussed in greater detail in Chapter 20, patients with PcP and respiratory failure that requires mechanical ventilation should ideally be treated with trimethoprim-sulfamethoxazole or pentamidine, which are both efficacious and can be administered intravenously, and with adjunctive corticosteroids. Careful attention must be paid to the parsimonious use of colloids and crystalloids so as to minimize a further decline in respiratory status from noncardiogenic pulmonary edema as a result of capillary leak and alveolar flooding. In patients who require mechanical

ventilation, ventilator strategies should follow current standards for patients with the adult respiratory distress syndrome [182].

The prognosis for HIV-infected patients with PcP requiring critical care has fluctuated over the course of the epidemic [174,183–197]. At SFGH, we have published a series of continuous reports documenting our ICU experience with patients with PcP throughout the AIDS epidemic [176–178,198–200]. From 1981 to 1985, only 14% of 42 persons with PcP and respiratory failure who required mechanical ventilation survived the hospitalization [198]. From 1986 to 1988, survival improved to 40% in the 35 patients who were reported [199]. The authors speculated that the improved survival was due to several factors, including the increased use of adjunctive corticosteroids compared to the previous era, greater experience in managing these patients, and potentially more careful screening of candidates for ICU admission and mechanical ventilation. However, from 1989 to 1991, survival worsened to 24% of 37 persons with PcP [200]. The precise reasons for the observed decrease in survival were unclear. Predictors of mortality were low CD4 lymphocyte counts on admission and the development of pneumothorax during mechanical ventilation. During the two most recent periods, from 1992 to 1995 and from 1996 to 2001, survival was 44 and 45%, respectively [176,178]. During this latter period, PcP was the only ICU admission diagnosis where the in-hospital survival was less than 50%; for all other admission diagnoses, the survival was at least 60%. Thus, it is sobering to realize that, despite all of our advances in the diagnosis and treatment of HIV infection and PcP and in the management of mechanically ventilated patients, HIV-infected patients with PcP and respiratory failure that requires mechanical ventilation are more likely to die than live.

The use of HAART may offer a new avenue to improve mortality from PcP-associated respiratory failure. One study that examined the impact of HAART on mortality in critically ill HIV-infected patients with PcP reported encouraging preliminary results [178]. In this retrospective study of 58 ICU patients with PcP, 12 patients (21%) were either receiving HAART or were started on HAART while hospitalized. Mortality in this group was 25%, compared to 63% in patients on no HAART ($p = 0.03$). Furthermore, multiple logistic regression analyses adjusting for potential confounders showed that HAART started either before or during hospitalization was associated with a lower mortality (odds ratio, OR, 0.14; 95% confidence interval, CI, 0.02–0.84; $p = 0.03$). The need for mechanical ventilation and/or development of a pneumothorax (OR, 20.9; 95% CI, 1.9–227.2; $p = 0.01$) and delayed ICU admission (OR, 9.7; 95% CI, 2.2–42.1; $p = 0.002$) were associated with increased mortality. While these results are certainly encouraging, additional studies (i.e., a prospective, randomized trial) are needed before firm recommendations can be made. Of note, there have been reports of immune reconstitution from HAART resulting in a paradoxical worsening of PcP

[201,202]. One report described three cases of acute respiratory failure after the initiation of HAART in patients who were successfully treated for PcP [201]. The patients all had severe PcP that initially improved with standard PcP and adjunctive corticosteroid therapy. HAART was introduced 1 to 16 days after diagnosis of PcP. Corticosteroids were stopped on day 15. Some 1 to 3 weeks after HAART introduction, the patients developed a second episode of severe acute respiratory failure, with high-grade fever and patchy alveolar opacities on the chest radiograph. *Pneumocystis* that was resistant to trimethoprim-sulfamethoxazole a subsequent respiratory infection, and drug-related pneumonitis were suspected. Bronchoalveolar lavage and lung pathological findings showed severe nonspecific pulmonary inflammatory foci surrounding a few persistent *Pneumocystis* cysts. All three patients recovered after HAART interruption or corticosteroid reintroduction. The authors postulated that this immune reconstitution phenomenon could result from rapid pulmonary recruitment of fully competent immune and inflammatory cells responding to a few persistent *Pneumocystis* cysts. In a critically ill patient with PcP and acute respiratory failure requiring mechanical ventilation, the potential risk of immune reconstitution must be balanced against the potential benefit of HAART.

B. Pneumothorax

PcP with an associated pneumothorax is perhaps one of the most challenging complications of HIV [203–208]. The combination of the acute pneumonia, the frequent presence of multiple pneumatoceles, and the use of adjunctive corticosteroids as a part of the PcP treatment all contribute to a difficult management problem with a high incidence of recurrence and bilateral lung involvement. Furthermore, PcP and acute respiratory failure that requires mechanical ventilation, with the subsequent development of a pneumothorax, is an almost universally fatal combination. In our experience at SFGH, only a few patients with these conditions have survived and the overwhelming majority of patients have died in the ICU.

Occasionally, patients will present with a small apical pneumothorax, which can be managed conservatively with oxygen therapy and close observation. More often, however, patients will present with a substantial collapse of a lung and will require urgent chest tube placement. The goal of chest tube placement is full expansion of the lung. The degree to which this is attainable depends on a number of factors, including the size of the pneumothorax, the length of time during which the lung has been collapsed, and the degree of lung compliance lost. Occasionally, the use of a chest tube will entrain a constant flow of air through a bronchopleural fistula (BPF), which will consequently limit healing. Various interventions have been used to produce closure of a BPF. At SFGH, we have a

conservative approach toward management of a BPF. The optimal treatment of the underlying PcP, the withdrawal (if possible) of adjuvant corticosteroids, optimization of the patient's nutritional status, and patience are the hallmarks of BPF management at this institution. If the lung remains expanded, pleurodesis should probably be attempted, as recurrent pneumothorax is common. The installation of a talc slurry into the pleural space incites pleural inflammation and, ideally, adhesion of the pleural surfaces. If successful, pleurodesis will prevent future pneumothorax. If only partially successful, the adhered pleural surfaces may prevent a recurrent pneumothorax from becoming a tension pneumothorax. Thoracoscopy with stapling has been performed with success for some selected patients. However, this procedure can be performed only in those patients able to tolerate the procedure who have a single BPF amenable to surgical closure. As a last resort, a patient may be discharged with a chest tube in place connected to a one-way valve (Heimlich valve) [209].

C. Chronic Airway Disease

After recovery from PcP, patients may develop chronic respiratory complaints. Although the development of chronic airway disease is certainly less dramatic than that of a pneumothorax, its effect on the patient can be equally profound. Pathologically, the destructive effects of PcP result in permanent damage to the airways. These unfortunate patients may develop symptoms identical to those of chronic bronchitis, with a chronic cough (especially in the morning) that is frequently productive [94]. They frequently become colonized with bacterial pathogens that are difficult or impossible to eradicate; as a result, recurrent bacterial infections are common and result in further airway destruction. In time, the cycles of infection and airway destruction may produce bronchiectasis. The vicious cycle of infection, treatment with broad-spectrum antibiotics, selection of increasingly resistant organisms, and recurrent pneumonia is a common scenario among patients with advanced HIV disease and prior PcP.

Despite successful PcP treatment, patients may note a permanent decline in their exercise tolerance. Morris and colleagues in the PCHIS found that PcP was associated with permanent decreases in forced expiratory volume in 1s (FEV_1), forced vital capacity (FVC), FEV_1/FVC , and the DL_{CO} [210]. This study modeled pulmonary function in 141 HIV-infected subjects who developed a first episode of PcP and compared them to 938 HIV-infected subjects without PcP or bacterial pneumonia. Multivariate modeling that controlled for predictors associated with pulmonary function—such as age, height, gender, race/ethnicity, HIV risk category, IDU, cigarette use, CD4 lymphocyte count, and time—found that PcP had a permanent negative impact on lung function. FEV_1 declined significantly after PcP (-264 mL, $p=0.001$), equivalent in magnitude to the estimated

effect of aging from 35 to 43 years. The FEV₁/FVC also declined significantly, and these declines appeared to continue to worsen over time. Clinically, many of these patients will respond to inhaled bronchodilators. In fact, one study found that HIV-infected patients developed bronchial hyperresponsiveness after an episode of PcP [211].

VI. Diagnosis

There is no universally agreed on approach to the management of HIV-infected patients with suspected PcP. Many institutions treat patients with suspected PcP empirically, while others, such as SFGH, pursue a definitive microscopic diagnosis. As *Pneumocystis* cannot be routinely cultured, the diagnosis of PcP relies on microscopic visualization of the characteristic cysts and/or trophic forms on stained respiratory specimens. Typically, these respiratory specimens are obtained from sputum induction or bronchoscopy. However, there is no universally agreed on diagnostic algorithm for the diagnosis of PcP, and institutions report different degrees of success with sputum induction and bronchoscopy with (BAL) with and without transbronchial biopsies (TBBx). Recently, advances in polymerase chain reaction (PCR) technology have enabled the use of oropharyngeal washing as a method for specimen acquisition. In the absence of prospective studies comparing various management and diagnostic strategies, the specific approach to a patient with suspected PcP is often based on the prevalence of PcP and clinician and institutional preferences and experiences [212–217]. The diagnostic algorithm used at SFGH is described here in order to highlight a number of the important considerations in the approach to the patient with suspected PcP.

A. Diagnostic Algorithm used at San Francisco General Hospital

At SFGH, we have used a diagnostic algorithm for suspected PcP with considerable success (Fig. 11) [94]. In our algorithm, patients with a clinical presentation suggestive of PcP who have chest radiographs with reticular or granular opacities undergo sputum induction. Induced sputum is sent for microscopic examination for *Pneumocystis* and acid-fast bacillus (AFB) smear and culture. At SFGH, the diagnostic sensitivity of sputum induction and Diff-Quik (modified Giemsa) staining for PcP has ranged from 74 to 81% [179,218]. This range of sensitivities indicates that 19 to 26% of patients with PcP will have negative induced sputum examinations for *Pneumocystis*; therefore patients with negative examinations undergo subsequent bronchoscopy with BAL unless an alternative diagnosis is established. BAL fluid is sent for microscopic examination for *Pneumocystis*,

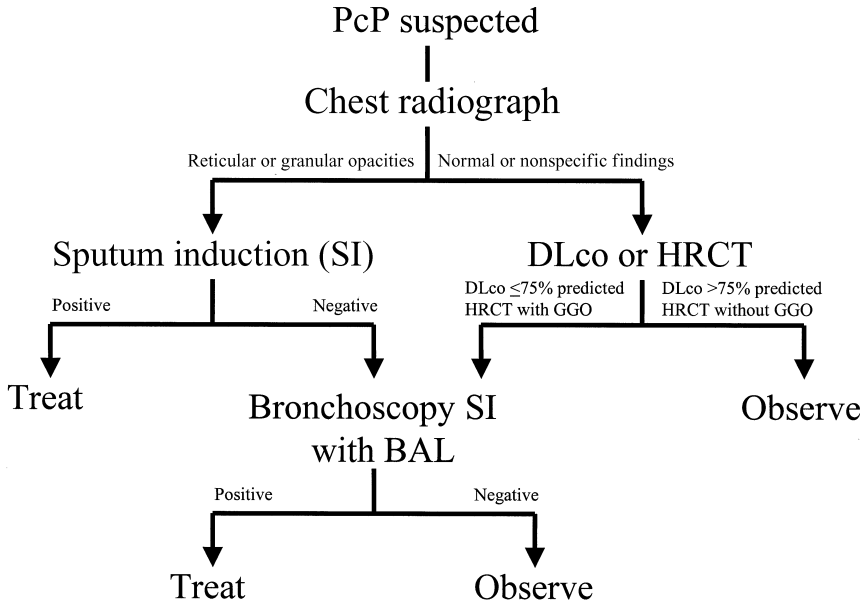


Figure 11 Diagnostic algorithm for the evaluation of patients with suspected PcP used at San Francisco General Hospital. DL_{co}, single-breath diffusing capacity for carbon monoxide; HRCT, high-resolution computed tomography; GGO, ground-glass opacities; BAL, bronchoalveolar lavage.

AFB smear and culture, and fungal culture. At SFGH, the diagnostic sensitivity of BAL and Diff-Quik staining for PcP approaches 100% [179]. Thus, TBBx are rarely if ever performed solely for the diagnosis of suspected PcP. At SFGH, clinical observation or evaluation and treatment for another pulmonary disease is recommended for patients whose BAL fluid examination is negative for *Pneumocystis* [94,179].

Patients with PcP may present with normal chest radiographs [129]. In our algorithm, patients with a clinical presentation suggestive of PcP who have normal chest radiographs undergo additional testing, either chest HRCT or pulmonary function testing. In these patients, further testing is used to distinguish which patients may have PcP and warrant sputum induction (and bronchoscopy if necessary) and which patients are unlikely to have PcP, such that diagnostic procedures for PcP and empirical PcP treatment are unnecessary. A prospective study reported that the combination of a normal or unchanged chest radiograph and a DL_{co} > 75% of predicted virtually ruled out PcP [19]. In this study, 101 of 103 consecutive HIV-infected patients with clinically suspected PcP, normal or

unchanged chest radiographs, and a $DL_{co} > 75\%$ of predicted had no evidence of PcP on sputum induction, bronchoscopy with BAL, or 30 days of clinical follow-up. As a result, we recommend that these patients either be clinically observed without further evaluation or treatment for PcP or be evaluated/treated for another process. One caveat: although a decreased DL_{co} is a sensitive test for PcP and therefore possesses a high negative predictive value, it lacks specificity for PcP. In this study, the sensitivity for PcP of 90% was accompanied by a specificity of 53% [19]. Therefore, a decreased DL_{co} cannot be interpreted as diagnostic of PcP, and patients with suspected PcP who have a decreased DL_{co} still undergo sputum induction in our algorithm.

A prospective study from SFGH found that the combination of a normal, unchanged, or equivocal chest radiograph and a chest HRCT without ground-glass opacities ruled out PcP [137]. In this study, no patient with suspected PcP, a normal, unchanged, or equivocal chest radiograph, and an HRCT without ground-glass opacity had a diagnosis of PcP established either on bronchoscopy or after 60 days of clinical follow-up. In my experience, no patient at SFGH has been diagnosed with PcP after having a normal chest radiograph and a chest HRCT without ground-glass opacities. Unfortunately, as in the case of a $DL_{co} \leq 75\%$ of predicted, an HRCT with ground-glass opacity lacks specificity for PcP, and persons with suspected PcP with these findings on HRCT must undergo sputum induction.

The coordinated effort of a number of services is vital to the optimal use of our diagnostic algorithm. First, clinicians initiate an evaluation and refer those patients with suspected PcP for sputum induction. Next, the Pulmonary Service screens all requests for sputum induction. The Pulmonary Service denies approximately 30 to 40% of all requests for sputum induction (in which case recommendations for further evaluation or treatment are provided) [219]. In one study, 30- and 60-day clinical follow-up of the 143 patients in whom a sputum induction request for PcP was denied confirmed the clinical accuracy of the Pulmonary Service in denying this request. In a cost analysis, the use of a Pulmonary Service to screen requests was shown to decrease the number of diagnostic procedures performed and to provide cost savings [220]. Those patients for whom the request is approved undergo sputum induction on the same day as the screening. A pulmonary laboratory technician closely supervises all patients undergoing sputum induction in order to obtain an appropriate specimen. In our experience and that of others, the use of experienced, dedicated personnel increases the number of successful attempts at sputum induction [221]. Finally, an experienced microbiology technician processes all specimens for *Pneumocystis* and systematically reviews each slide with a microbiologist. In our experience, definitive diagnosis is preferable to empirical therapy. The coordinated effort described allows the pursuit of a definitive diagnosis using sputum induction as the initial procedure

and reserving bronchoscopy for patients whose induced sputum examination is negative for *Pneumocystis*.

B. Sputum Induction

Sputum specimens may be used to diagnose PcP. It may also diagnose other important HIV-associated opportunistic infections. Occasionally, patients with PcP present with a productive cough, usually with clear to white sputum. A few studies have examined the sensitivity of spontaneously expectorated sputum for the diagnosis of PcP and report a diagnostic sensitivity of 52 to 55% [222,223]. However, most patients with PcP present with a dry, nonproductive cough and invariably require sputum induction to produce suitable specimens. Sputum induction is a safe, simple, and relatively well-tolerated procedure [224]. Patients should have taken nothing by mouth (NPO) for 6 to 8 hrs prior to induction. Before induction, patients cleanse the oral cavity and rinse and gargle with normal saline to remove food particles, bacteria, and epithelial cells that might interfere with microscopic examination. Sputum is induced by the inhalation of hypertonic saline generated by an ultrasonic hand-held nebulizer. Induction is continued for 15 to 20 min or until a specimen volume of 5 to 10 mL is produced (collected into two separate containers). Patients often experience a transient (<20-min) decrease in their oxygen saturation [225]. Rarely, coughing may be so severe as to cause vomiting (affirming the importance of being NPO). There have been numerous studies that have examined the sensitivity of induced sputum for the diagnosis of PcP, several of which are highlighted below.

The sensitivity of sputum induction for PcP depends on several factors, including the method for preparing the specimen and the specific staining technique used. Stains such as Gomori methenamine silver (GMS), toluidine blue O, and cresyl echt violet stain the wall of *Pneumocystis* cysts, while Wright-Giemsa and Diff-Quik (a modified Giemsa) stain the nuclei of the trophic forms and cysts. Calcofluor white is a chemifluorescent agent that nonspecifically binds to β -linked polysaccharide polymers of fungi and *Pneumocystis*. The first reports of sputum induction for PcP were performed by direct smear onto a slide before staining [226,227]. These two initial studies reported a sensitivity of sputum induction for PcP of 56 and 55% using GMS and Giemsa staining, respectively. A subsequent study that used sputum liquefaction with dithiothreitol and then specimen concentration with centrifugation reported a significantly higher sensitivity for PcP of 78% [228]. In this study, specimens from concentrated sputum were more sensitive for PcP (78%) than were specimens from unconcentrated sputum (43%). Subsequent studies demonstrated that the sensitivity of sputum induction for PcP is further increased with the use of direct and indirect immunofluorescent stains using monoclonal antibodies for *Pneumocystis* [216,229–236].

One study reported that immunofluorescent staining (92%) was significantly more sensitive than toluidine blue O (80%) and Diff-Quik/Giemsa staining (76%) [229]. Finally, studies have shown that the sensitivity of induced sputum for PcP is also excellent with the use of PCR-based tests [237–241]. In one study, PCR on induced sputum was positive in 90% of patients with PcP, compared to only 35% for GMS staining [237]. However, the specificity of these PCR-based tests has been reported to be less than 100% and patients without clinical PcP have had false-positive PCR results; presumably these patients are colonized with *Pneumocystis*.

The prevalence of PcP at different institutions was postulated to affect the sensitivity of sputum induction for PcP. However, studies from cities that had different numbers of patients with HIV/AIDS and PcP found comparable results using the same methods [218,242]. In a 10-month study from San Francisco, 287 HIV-infected patients were diagnosed with PcP, and the sensitivity of sputum induction for PcP was 77% using Diff-Quik [218]. After a tutorial in San Francisco, a 14-month study from Denver reported that 38 patients were diagnosed with PcP, and the sensitivity of sputum induction for PcP was 66% using Diff-Quik [242]. In a 4-month study from London that closely followed the San Francisco protocol, 19 HIV-infected patients were diagnosed with PcP, and the sensitivity of sputum induction for PcP was 95% [243].

The severity of PcP is reported to have no effect on the sensitivity of sputum induction for PcP [244]. In this study, patients with PcP diagnosed by sputum induction were similar to patients with PcP diagnosed by BAL after negative sputum induction with regard to serum LDH, arterial oxygen tension, and chest radiographic score.

The PcP episode (first or recurrent) being evaluated is reported to have no effect on the sensitivity of sputum induction for PcP [245]. In this study, the sensitivity of sputum induction for PcP was 72% for the first episode of PcP and 72% for the second episode. In addition, sputum induction can be used in patients with recurrent PcP whose initial diagnosis was missed on induced sputum and established by bronchoscopy. One study found that the sensitivity of sputum induction for PcP was 72% for patients with a second PcP episode whose initial PcP was detected by induced sputum and 71% for patients with a second PcP episode whose initial PcP was missed by induced sputum [245].

Most of the studies that examined the effect of aerosolized pentamidine prophylaxis on the sensitivity of sputum induction for PcP found no difference between patients receiving this therapy for prophylaxis and those on no prophylaxis [163,164,246,247]. Although there are no published studies on the effect of oral prophylaxis (trimethoprim-sulfamethoxazole, dapsone, and atovaquone) on the sensitivity of sputum induction for PcP, the clinical experience at SFGH suggests that there is no difference.

Clinically, it is important to recognize that *Pneumocystis* can be detected in sputum specimens weeks after the initiation of successful PcP treatment [248]. In one study, *Pneumocystis* was detected on repeat induced sputum specimens using immunofluorescent staining in 88% of patients at 2 weeks and 76% at 3 weeks after PcP diagnosis (at completion of a standard 21-day course of PcP treatment). Nearly 30% of patients had residual *Pneumocystis* detected at 4 weeks and almost one-quarter had *Pneumocystis* detected at 6 weeks. As a result, the diagnosis of recurrent or relapsed PcP within a 2- to 3-month period of the initial diagnosis is a clinical diagnosis and cannot be established microscopically.

C. Bronchoscopy

Bronchoscopy with BAL is considered to be the “gold standard” diagnostic test for PcP. Bronchoscopy with BAL using a flexible fiberoptic bronchoscope is a simple and well-tolerated procedure. As for sputum induction, patients should be NPO for 6 to 8 hrs prior to the procedure. Patients are given topical anesthesia and occasionally intravenous narcotics and benzodiazepines. In patients whose chest radiographs reveals diffuse, symmetrical disease, BAL is usually performed in the right middle lobe. Anatomically, a BAL from this lobe provides the greatest yield. However, in patients whose radiograph reveals focal or asymmetrical disease, performing BAL in the lobe with the greatest radiographic involvement or performing a bilobe lavage (both in the lobe with the greatest radiographic involvement and the right middle lobe) should be considered [249–252]. BAL is performed by wedging the bronchoscope in a subsegmental bronchus. Typically, four to five sequential 20- to 25-mL aliquots of sterile, nonbacteriostatic saline are instilled and aspirated into a sterile specimen container.

Bronchoscopy with BAL is a sensitive procedure to diagnose PcP [179,231,253–269]. Early in the HIV/AIDS epidemic, bronchoscopy usually included both BAL and TBBx [254–256]. Alone, each procedure had a reported sensitivity for PcP $\geq 85\%$; combined, the sensitivity of bronchoscopy ranged from 94 to 100%. Subsequently, bronchoscopy with BAL alone was found to be a sensitive procedure to diagnose PcP [258,259]. In these studies, the sensitivity of bronchoscopy with BAL for PcP was found to be $\geq 97\%$. The high sensitivity of BAL combined with the complications associated with TBBx (pneumothorax) led many institutions to perform BAL alone as the initial bronchoscopic test in patients with suspected PcP. Additionally, BAL may also diagnose other important HIV-associated opportunistic infections. Two large series reported that BAL can provide a diagnosis of bacterial, mycobacterial, and fungal pneumonias [179,267]. In a series from Cincinnati, Baughman and colleagues performed a total of 894 bronchoalveolar lavages on HIV-infected patients over a 7-year period [267]. Bronchoalveolar lavage provided a diagnosis in 530 (60%) cases. In addi-

tion to PcP ($n = 420$), BAL was able to diagnose *Mycobacterium tuberculosis* ($n = 17$), *Mycobacterium kansasii* ($n = 15$), *Histoplasma capsulatum* ($n = 24$), *Cryptococcus neoformans* ($n = 17$), and bacterial infection ($n = 103$). For 364 lavages, no microbiological diagnosis was made. In a study from San Francisco, Huang and colleagues examined a total of 602 bronchoscopies on HIV-infected patients with suspected PcP over a 4-year period [179]. These patients had a negative induced sputum examination for *Pneumocystis* and underwent subsequent bronchoscopy. Overall, bronchoscopy with BAL provided a diagnosis in 299 (50%). These diagnoses included tracheobronchial Kaposi's sarcoma lesions seen during bronchoscopic examination [180,270] as well as PcP ($n = 192$), *M. tuberculosis* ($n = 28$), *C. neoformans* ($n = 9$), *H. capsulatum* ($n = 6$), *Coccidioides immitis* ($n = 3$), *Legionella pneumophila* ($n = 3$), *Strongyloides stercoralis* ($n = 2$), and one case each of *Nocardia asteroides* and *Toxoplasma gondii* pneumonias.

In contrast to a negative induced sputum examination, a negative BAL fluid examination for *Pneumocystis* may be sufficient to rule out a diagnosis of PcP [179,266]. A SFGH review of 100 HIV-infected patients with suspected PcP who had a negative BAL fluid examination for *Pneumocystis* and had no PcP therapy identified no cases of subsequent PcP within 30 days of the initial negative evaluation [179]. Two cases of PcP were diagnosed between 30 and 60 days, one case after 46 days, the other after 51 days from the initial negative evaluation. Thus, a negative BAL fluid examination for *Pneumocystis* may obviate the need for more invasive procedures (i.e., TBBx). However, in populations of HIV-infected persons with a high background incidence of *M. tuberculosis* infection, the diagnosis of tuberculosis may be enhanced by the use of TBBx.

Bronchoscopy with BAL is also a sensitive procedure to diagnose PcP in infants and children [97,271–278]. In several small series, the sensitivity of bronchoscopy with BAL has been 100%, and none of the patients with a negative BAL fluid examination for *Pneumocystis* was found to have PcP on clinical follow-up [273,275]. As for adults, the use of bronchoscopy with BAL decreases the need for the more invasive TBBx and open lung biopsy.

Studies that examined the effect of aerosolized pentamidine prophylaxis on the sensitivity of bronchoscopy with BAL for PcP have found differing results [247,250–252,279]. Some studies found no difference in diagnosis between patients receiving this therapy for prophylaxis and those on no prophylaxis, while others have found a higher yield from BAL performed in the upper lobe compared to the middle or lower lobe. One study found that the number of *Pneumocystis* “trophozoites” was higher in patients with PcP who were receiving prophylaxis than in those on no prophylaxis [280]. In addition, the ratio of cysts to trophozoites was lower in patients with PcP who were receiving prophylaxis. No difference was observed between patients receiving different prophylactic medications. The

authors postulated that the imbalance between *Pneumocystis* forms in BAL fluid from patients with PcP receiving *Pneumocystis* prophylaxis may hamper the sensitivity of cyst stains. Another study found no correlation between the number of *Pneumocystis* organisms and the clinical course or the outcome of PcP [281]. Given these results, it is probably prudent to perform a bilobe lavage in patients with suspected PcP whose chest radiographs reveal focal or asymmetrical disease.

Pneumocystis can be detected in bronchoscopy specimens weeks after the initiation of successful PcP treatment [282–287]. As a result, the diagnosis of recurrent or relapsed PcP within a 2-to 3-month period of the initial diagnosis is a clinical diagnosis and cannot be established microscopically.

D. Other Tests

Nonbronchoscopic procedures can also diagnose PcP. Nonbronchoscopic lavage has been used [288–292]. For mechanically ventilated patients, endotracheal and nonbronchoscopic lavage have been used [293–295]. One study compared the insertion of a 14F catheter into the trachea under local anesthesia to standard bronchoscopy [290]. In this study, tracheal catheterization was able to diagnose 35 of the 40 (88%) cases of PcP diagnosed by BAL. Transthoracic needle aspiration can also be used to diagnose PcP [296–298]. In select patients, open lung biopsy has established a diagnosis of PcP that was previously missed [299,300].

Recently, the measurement of plasma S-adenosylmethionine concentrations was found to be a sensitive test for PcP [301]. S-adenosylmethionine is an important biochemical intermediate in many cellular functions, and it has been shown to be depleted in laboratory animal studies of PcP. In one study, plasma S-adenosylmethionine concentration was undetectable in 14 of 15 HIV-infected patients with either histologically confirmed or clinically suspected PcP and the median S-adenosylmethionine concentration in these patients was significantly lower than that in healthy control subjects or HIV-infected patients without PcP [301]. In addition, serial S-adenosylmethionine concentrations appeared to parallel the clinical course of PcP treatment in these patients. Three PcP patients who clinically responded to treatment had a rise in their S-adenosylmethionine concentrations to the normal range. However, further studies are warranted to confirm these initial results.

PCR technology has allowed for the potential use of specimens obtained noninvasively from oropharyngeal washing (i.e., gargling) to diagnose PcP. Several studies that compared a PCR assay from oropharyngeal washing specimens to standard microscopy from induced sputum or BAL specimens have reported promising results [302–307]. These studies report that PCR from oropharyngeal washing specimens has a diagnostic sensitivity for PcP ranging from 79 to 94%. Unfortunately, the specificity of these PCR-based assays is often less than 100%

(patients without PcP may have a false-positive PCR test result), which is a significant limitation, given the significant adverse effects and toxicities from PcP treatment regimens. Studies are ongoing to develop approaches that can distinguish patients with active pneumonia from those who are merely colonized with *Pneumocystis* and to determine the clinical significance of *Pneumocystis* colonization.

VII. Selected Issues in Treatment

HIV-infected patients with suspected PcP should be started on treatment immediately. Whether all patients should undergo diagnostic procedures to establish a microscopic diagnosis of PcP is debated [308–316]. One study successfully used empirical PcP treatment [308]. In this study, the use of empirical PcP treatment for typical PcP led to the correct treatment in 43 of 45 cases (95%) and would have saved 44 of the 45 bronchoscopies in this group. Overall, 44 of 69 bronchoscopies (64%) would have been saved, and the sensitivity and specificity for the diagnosis of PcP was 85%. In contrast, other studies have found a worse outcome associated with empirical PcP treatment. In a study of 227 patients, the mortality for the 47 empirically treated episodes was significantly higher than that for the 180 microscopically diagnosed episodes [309]. Another study examined cases of PcP in three U.S. cities [311]. This study found that persons who were empirically treated for PcP had higher mortality rates than those who had a bronchoscopy, regardless of whether the bronchoscopy demonstrated *Pneumocystis*. The authors postulated that the use of bronchoscopy may have contributed to better outcomes for persons treated for HIV-related PcP by diagnosing other HIV-associated opportunistic infections. One study linked insurance status to the use of bronchoscopy and the outcome of PcP [312]. This study found that Medicaid patients were less likely to undergo bronchoscopy than privately insured or self-insured patients, were more likely to be empirically treated for PcP, and were more likely to die in hospital.

In my opinion, empirical therapy should be approached with caution, and appropriate patients should be selected carefully. A number of criteria should be met before empirical therapy is begun. First, the patient must be at risk for PcP, usually on the basis of a CD4 lymphocyte count $<200/\text{mm}^3$ or a prior history of PcP. Next, the patient should not be on *Pneumocystis* prophylaxis, especially trimethoprim-sulfamethoxazole. Since the development of PcP while on this combination is infrequent, clinicians who are presented with such patients should make every effort to find an alternate diagnosis before embarking on a course of empirical therapy. Third, the patient should have a clinical and radiographic presentation strongly suggestive of PcP. The presence of atypical features should

prompt a consideration of alternate diagnoses. Furthermore, the patient should have mild disease. The potential consequences of a missed or delayed diagnosis in a patient with severe respiratory dysfunction argue strongly for the pursuit of a definitive diagnosis in most or all cases. Next, the patient should be reliable, compliant with medications, able to tolerate the proposed therapy (without nausea/vomiting, diarrhea, or a history of previous adverse drug reactions to that therapy), able to recognize the early progression of symptoms, and willing to return for follow-up visits. In patients fulfilling these criteria, empirical therapy may be a suitable alternative to invasive diagnostic procedures. However, if there is clinical progression or even a slow clinical response to empirical therapy, diagnostic tests and procedures should be pursued.

VIII. Conclusion

This chapter reviews the incidence of PcP both as an AIDS-defining diagnosis as well as in cohorts of HIV-infected patients. Although the incidence of PcP has declined dramatically in the United States, western Europe, and areas of the world where combination antiretroviral therapy is widely available, PcP is increasingly described in Africa, Asia, and Latin America, areas of the world where greater than 90% of the estimated people living with HIV/AIDS reside and where access to combination antiretroviral therapy and even *Pneumocystis* prophylaxis is limited. Improved surveillance in these areas is critical. In the United States and western Europe, continued surveillance for PcP, especially in patients failing HAART therapy due to multi-drug-resistant HIV will be vital, as PcP will presumably herald the onset of any HAART-resistant HIV epidemic.

This chapter discusses risk factors for PcP, chiefly the CD4 lymphocyte count and, to a lesser extent, the HIV RNA. Despite advances in our current understanding of risk factors for PcP, the majority of cases of PcP occur in persons without prior medical care and without a prior known HIV diagnosis. Among patients with a known HIV diagnosis who are in medical care, patient nonadherence with prescribed *Pneumocystis* prophylaxis and use of a prophylactic regimen other than trimethoprim-sulfamethoxazole account for the majority of the PcP cases seen. An improved understanding of the natural reservoir of human *Pneumocystis* and the potential of person-to-person transmission might provide new avenues for disease prevention.

This chapter also describes the clinical and radiographic presentation of PcP, the use of tests such as chest HRCT and pulmonary function testing in the evaluation of suspected PcP, and the algorithm used at San Francisco General Hospital for the diagnosis of PcP. Classically, PcP presents with fever, cough, and dyspnea on exertion and bilateral, diffuse, symmetrical reticular (interstitial)

or granular opacities on chest radiograph. There is no universally agreed on approach to the management of HIV-infected patients with suspected PcP. Many institutions treat patients with suspected PcP empirically, while others, such as SFGH, pursue a definitive microscopic diagnosis. Both sputum induction and bronchoscopy with BAL are sensitive procedures for the diagnosis of PcP. Future studies to define the optimal approach to a patient with suspected PcP, including the use of noninvasive oropharyngeal wash specimens that can be used in resource-limited settings, are essential.

List of Abbreviations

ABG:	arterial blood gas
AFB:	acid-fast bacillus
ASD:	Adult and Adolescent Spectrum of HIV Disease
BAL:	bronchoalveolar lavage
BOOP:	bronchiolitis obliterans organizing pneumonia
BPF:	bronchopleural fistula
CDC:	Centers for Disease Control
DL _{co} :	diffusing capacity for carbon monoxide
FEV ₁ :	forced expiratory volume in 1 sec
FVC:	forced vital capacity
GMS:	Gomori methenamine silver
HAART:	highly active antiretroviral therapy
HIV:	human immunodeficiency virus
HOPS:	HIV Outpatient Study
HRCT:	high-resolution computed tomography
ICU:	intensive care unit
IDSA:	Infectious Diseases Society of America
IDU:	injection drug users
LDH:	lactate dehydrogenase
MACS:	Multicenter AIDS Cohort Study
MSM:	men who have sex with other men
NIH:	National Institutes of Health
NPO:	Nothing by mouth
PCHIS:	Pulmonary Complications of HIV Infection Study
P ² C ² HIV Study:	Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted HIV Infection Study
PcP:	<i>Pneumocystis pneumonia</i>
PCR:	polymerase chain reaction
PFT:	pulmonary function test
SFGH:	San Francisco General Hospital
TBBX:	transbronchial biopsies
USPHS:	U.S. Public Health Service
WHO:	World Health Organization

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***Pneumocystis* Pneumonitis in Non-HIV-Infected Patients Update**

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This chapter focuses on the assessments and developments of *Pneumocystis* pneumonia (PcP) in non-AIDS patients during the past decade, supplementing the information provided in the previous edition of this book and other texts.

I. Comparison of *Pneumocystis* Pneumonitis in Non-AIDS and AIDS Patients

Prior to the onset of the AIDS epidemic in 1981, PcP occurred almost exclusively in immunocompromised patients with cancer, organ transplant recipients, those with congenital immunodeficiency disorders, and debilitated infants in Europe. The foundation of our clinical knowledge of PcP came from the study of such patients. As the prevalence of AIDS escalated and the high incidence of 60 to 70% for PcP was recognized, attention shifted to the problem in HIV-infected patients, leading to a dearth of new data related to PcP in non-AIDS patients over the ensuing two decades. Nevertheless, the population without AIDS at high risk for PcP has increased, and in some instances new risk categories have been recognized. It is reasonable to ask whether data from studies of PcP in patients with cancer and organ transplants before 1980 are fully applicable to such patients today. New drugs and biological agents for treatment of the primary diseases as well as new drugs for the treatment and prevention of PcP have come into use.

How do patients with PcP and AIDS differ from those with PcP and non-AIDS immunocompromising diseases? Can information gained from studies of PcP in one group be applied to the management of patients in the other?

At the beginning of a new millennium, significant changes are apparent with respect to PcP in both AIDS and non-AIDS patients. The application of PcP chemoprophylaxis and highly active antiretroviral therapy (HAART) in management of HIV infections has resulted in a striking decrease in the prevalence of PcP in AIDS. Currently, for patients diagnosed with HIV infection in the United States and managed by standard-of-practice guidelines, the incidence of PcP is about 5.2% per 100 person-years [1]. During the decade from 1990 through 1999, PcP cases in England and Wales diminished in men from 2.77 to 0.42 cases per million population, but with little change in women [2].

Although the incidence of PcP in immunocompromised patients without AIDS is much lower than among those with AIDS, the population of the former is much greater and is increasing. For example, approximately 1,268,000 new cases of cancer were diagnosed in the United States in 2001, more than 30,000 patients undergo bone marrow transplants each year, and more than 20,000 individuals receive solid organ transplants annually [3], but only 42,000 new cases of AIDS are diagnosed yearly.

Comparison of 89 HIV-positive and 32 HIV-negative immunocompromised patients with PcP occurring from 1983 through 1998 at the Basel University Hospital in Switzerland revealed some differences in clinical features and changes over time [4]. The duration of symptoms was longer in AIDS patients (23 vs. 13 days; $p < 0.005$), and a higher frequency of weight loss, sweating, and thoracic pain (16 vs. 31%; $p < 0.05$) occurred with AIDS. Mortality rates were similar. In the non-AIDS patients, the mortality rate decreased from 29 to 7% ($p < 0.001$) after 1995 and hospital stay 34 to 16 days ($p = 0.005$). This improvement in survival was attributed to increasing experience in the treatment of opportunistic infections in AIDS.

Useful information came from the comparison of 131 HIV-infected patients and 86 patients without HIV at the time of diagnosis of PcP in Northern California [5]. The overall outcome of PcP was worse in the non-AIDS patients, but recurrence of the pneumonitis was less and tolerance was better to trimethoprim-sulfamethoxazole (TMP-SMX) in this group. In the non-AIDS group, PcP occurred in 8 patients during 1980–1984; 23 patients during 1985–1989; and 51 patients during the period from 1990 to 1994. This increase in PcP was attributed to the use of stronger immunosuppressive regimens, more awareness of the need to search for PcP, and improved diagnostic methods. It is important to note that the median CD4 lymphocyte counts in the non-AIDS group was 174/mm³ and the median cell count in the AIDS group was 50/mm³.

The outcomes of the first episodes of PcP in 58 HIV-infected patients were compared to those of 16 non-AIDS patients in Germany in 1995 [6]. The mortality

rate in the HIV group was closely related to the severity of PcP and the HIV infection. The types of malignancy and collagen vascular disease accounted for the high mortality rate in the non-AIDS group. The only variable found to be significantly associated with death in both groups was low serum albumin values. Protein-calorie malnutrition, reflected by low serum albumin, has been identified as a provocative risk factor for PcP in both human and animal studies [7]. Mansharamani et al. [8] analyzed the outcomes of 605 adults with AIDS and 33 cases of non-AIDS with PcP during 1985–1995. The mortality rate for the AIDS-related cases fell from 11.7% to 6.6%, while the mortality rate for the non-AIDS cases was 39%.

The recent comparative studies of AIDS and non-AIDS patients with PcP [4,5] report results similar to those of Kovacs et al. [9] in 1984.

II. High-Risk Categories for *Pneumocystis* Pneumonia in Non-AIDS Patients During the Past Decade

Risk categories for PcP have changed by decades over the past half century or so. During the 1940s and 1950s, PcP was found predominately in debilitated European infants. Within the decade beginning in 1960, immunosuppressive anti-cancer therapy came into greater use and cancer patients became the major risk group. By 1970, organ and bone marrow transplantation was gaining widespread application and added another population of high-risk individuals for PcP. From 1980 on, HIV-infected individuals composed the major entity with predisposition for PcP.

In dealing with the current issue of PcP in non-AIDS patients, the prevalence of underlying high-risk conditions during the past decade is of interest. Specialty hospitals such as cancer treatment institutions and organ transplantation centers provide information only for their limited clientele. Perhaps more meaningful data come from studies of PcP in a general hospital setting. Two such studies are summarized in Table 1: one study from the University Hospital in Lieden in 1995 [10] and another from the Basel University Hospital in Basel in 1999 [4]. The comparison shows a similarity of conditions at the two medical centers in that both sites experienced PcP in patients with malignancies, systemic vasculitis/autoimmune diseases, and organ transplantation, although the relative prevalence of these varies, probably due to the type and number of immunosuppressed patients admitted to the respective hospitals.

In comparison to studies prior to the AIDS epidemic, the Leiden and Basel studies show the emergence of patients with transplantation and also the group of conditions designated systemic vasculitis/autoimmune disease. These investigators as well as others [11–15] observed an increasing number of PcP cases during the period of study.

Table 1 Predisposing Factors in Patients with *Pneumocystis* Pneumonia without AIDS in General Hospitals

Underlying disorder	University Hospital Leiden, 1995 (10)	University Hospital Basel, 1999 (4)
	No. (%) of Patients <i>N</i> = 78	No. (%) of Patients <i>N</i> = 32
Hematologic malignancy	38 (49%)	3 (9%)
Solid tumors	3 (4%)	—
Systemic vasculitis/autoimmune	17 (22%)	4 (13%)
Renal transplantation	13 (17%)	19(59%)
Bone marrow transplantation	7 (9%)	5 (16%)
Idiopathic CD4 cell deficiency	—	1
Total	78 (100%)	32 (100%)

Review of cases of PcP in non-AIDS patients at the Mayo Clinic in 1996 [16] found 116 cases in patients with hematological malignancies, solid tumors, organ transplantation, inflammatory diseases, and other conditions during a 6-year period. Over 90% of the patients received corticosteroid drugs within the month prior to PcP and 85% were receiving these drugs at the time of diagnosis.

A reasonable premise would suggest that the risk for PcP is related directly to the extent of immunocompromise of the host, especially cell-mediated immunity. The risk is more the extent of immunosuppression than the specific underlying entity that predisposes to PcP. The CD4 T-lymphocyte count or percentage is a reasonably dependable indicator of the host's susceptibility to PcP.

A. Cancer

Update

Some indication of differences in PcP in patients with cancer during the past decade and earlier years is provided by retrospective review of patients at Memorial Sloan-Kettering Cancer Center. A total of 52 histologically proven cases of PcP from 1990 to 1994 were compared to 142 cases from 1978 to 1989 [11,17]. Corticosteroid use was a risk factor in 71% of the cases before 1990, compared to 87% of those evaluated in 1990 and later ($p<0.03$). Of the 15 patients not on corticosteroids, the risk factors for PcP were bone marrow transplantation in 6, lymphoma in 4, fludarabine treatment of chronic lymphogenous leukemia in 3, severe combined immunodeficiency syndrome in 1, and high-dose cytoxan in 1. The survival rate with PcP was 75% for the cases in the 1990s, compared to 51% for those before 1990 ($p<0.007$). The 2002 report of 10 years' experience from GIMEMA centers in Europe reveals 52 cases of PcP during the decade from

1900 to 1999. Only 2 of the patients received prophylactic TMP-SMX. The mortality rate was 29%. Prolonged corticosteroid therapy was a significant risk factor [18].

The introduction of fludarabine, a new antimetabolite, approved in 1991 for general use, represents a significant advance in the treatment of chronic lymphocytic leukemia (CLL). This low-grade lymphoid malignancy has rarely been associated with PcP. However, in 1995 Byrd et al. [19] observed PcP in 4 (25%) of 21 alkylator-resistant CLL patients treated with fludarabine. A literature review of 2269 patients with low-grade lymphoid malignancies who received fludarabine therapy revealed that only 24 (1%) cases of PcP occurred. Interestingly, all of the 24 PcP cases of Byrd et al. had received an alkylator drug or corticosteroids related to fludarabine therapy. Clinicians must now consider PcP prophylaxis in this new group of patients at risk.

At the beginning of the 1990s, reports began to appear of PcP in patients with brain tumors. The first detailed descriptions in 1991 by Henson [20] described 10 patients with primary brain tumors at Memorial Sloan-Kettering Cancer Center. In 8 of these patients, the pneumonitis occurred during tapering of corticosteroid doses. In 1993, Slivka et al. [21] reported 4 patients with PcP and intracranial neoplasms who also developed the pneumonitis during the corticosteroid taper. The nine patients with brain tumors at the Mayo Clinic reported by Schiff [22] were receiving corticosteroids at the time of PcP.

Although breast cancer is one of the most common malignancies, accounting for 27% of all cancers in women, PcP has not frequently been associated with this malignancy. Kulke and Vance [23] found only 24 cases of PcP in patients with breast cancer prior to 1997. They reported two cases with metastatic breast cancer treated with high doses of cyclophosphamide and blood stem cell infusion. Siminske et al. [24] described 3 cases of PcP and breast cancer during an 18-month period in Seattle, and Koibuchi et al. [25] reported an additional case in 1995 from Japan. The case of Taattevin et al. [26] occurred after a single course of low-dose nonmyeloablative chemotherapy and local radiotherapy. The changes in the type and intensity of chemotherapy, methods for procurement of autologous stem cells, and use of bronchoalveolar lavage for the diagnosis of pneumonitis have been suggested as factors in the increase of PcP in breast cancer patients.

The results of an intensive five-drug remission induction regimen with intensive consolidation for adults with acute lymphoblastic leukemia from the Cancer and Leukemia Study Group B (CALGB) were reported in 1995 [27]. Eleven (5.5% of the 197 patients) were diagnosed with PcP at some point during the 2 years of therapy. It is noteworthy that in this study, PcP prophylaxis with TMP-SMX or aerosolized pentamidine was not recommended until after the 3 months of induction and intensification phases of chemotherapy.

Investigators from several cancer centers report that the incidence and/or prevalence of PcP in patients with cancer is increasing over the past decade

[9–11,17,28,29]. The prevalence of PcP in non-AIDS patients has been tabulated at the University Hospital of Leiden in the Netherlands. From 1981 through 1984, 12 cases of PcP were diagnosed; from 1985 through 1989, 25 cases were admitted; and from 1990 through 1993, 41 cases occurred. A similar trend was found at the University Hospital in Basel [4] from 1988 through 1998.

Clinical Aspects

The predominant clinical manifestations of PcP are fever, cough, tachypnea, and diffuse bilateral pulmonary infiltrates by chest radiography. Of 17 cases of PcP reported in 1992 in cancer patients at the Institut Jules Bordet, 88% had fever, 76% had cough, 59% had shortness of breath; alveolar and interstitial infiltrates were found in 94% of the cases. Arterial oxygen tension was < 75 mmHg in 91% of patients tested [12]. Of the 29 adults with acute lymphoblastic leukemia (ALL) reported by Lyytikainen et al. [28] in 1996, 7 (24%) developed PcP. All the patients had respiratory symptoms, hypoxia, and infiltrates in chest radiographs. Of the 7 episodes, 4 occurred from 19 days to 2 months and 3 from 2 to 33 months after starting anticancer therapy.

Diagnosis

Demonstration of *Pneumocystis* cysts, trophic forms, or both in pulmonary tissue or secretions is required for a definitive diagnosis. Material for study is obtained by induced sputum, bronchoalveolar lavage (BAL), or transbronchial or open lung biopsy.

BAL is usually recommended as the initial method unless the organism is found in induced sputum. Sputum samples, even when available, often do not contain the organism, especially in non-AIDS patients, where the magnitude of infection is usually lower than in AIDS patients. Based on study of AIDS patients, the diagnosis can be made from first-time BAL specimens in about 80% of cases. If a second procedure is done in negative cases, the overall sensitivity of the procedure is enhanced. BAL is less invasive and more safe than biopsy methods. Clinicians with experience with PcP in both AIDS and non-AIDS patients are of the opinion that the number of organisms is fewer in the non-AIDS patients, and BAL yields may show fewer diagnostic forms. Also, the procedure may be more difficult in infants and small children than in adults.

The most sensitive and specific method for diagnosis is open lung biopsy, which provides a diagnostic specimen in more than 95% of cases. Open lung biopsies or transbronchial biopsies are usually used when the BAL technique has failed to establish the diagnosis in highly suspected cases. A recent report from Memorial Sloan-Kettering Cancer Center found that all of their cases of granulomatous PcP required open lung biopsy for diagnosis after negative BAL studies [29]. It should be noted that of all the specimens used for diagnosis, only the

lung biopsy permits the diagnosis of **pneumonitis** due to *Pneumocystis*. Other specimens yield only the organism and do not differentiate a carrier state from active pneumonitis. Nevertheless, other clinical features—such as pulmonary infiltrates by radiograph in an immunocompromised host plus identification of the organism in BAL or sputum—permits an accurate diagnosis.

Preliminary studies have shown that *P. carinii* f. *Sp. hominis* DNA detected by polymerase chain reaction (PCR) from BAL and nasopharyngeal aspirates may serve as an indicator of PcP in cancer patients [30,31]. More extensive studies in AIDS patients offer optimism for the further development of this method for diagnostic purposes. However, at present it is available only at the investigative level, pending eventual standardization of methods and commercial availability.

Treatment

The treatment of choice for PcP is trimethoprim-sulfamethoxazole (TMP-SMX). For patients who cannot take TMP-SMX or who fail treatment with this regimen, intravenous pentamidine isethionate may be used. Several other drugs and drug combinations have been successful in the treatment of PcP in AIDS patients but have not been adequately evaluated in patients with cancer. These include TMP-dapsone [32]; atovaquone [33]; and pyrimethamine plus clindamycin [34]. See chapters 15 and 19 on AIDS and treatment for further details.

The use of adjuvant corticosteroids in the treatment of severe PcP in adults with AIDS has been shown to increase survival rates. A recent study by Pareja et al. [35] evaluated two dose levels of prednisone, ≥ 60 or ≤ 30 mg, in 31 non-AIDS adults with PcP. Although intubation and mortality rates were similar, recovery was accelerated in those receiving the high dose. In another study by Delclaux et al. [36] of similar size, adjunctive corticosteroid therapy did not improve survival. A short course of a few days of prednisone for moderately severe and severe cases of PcP is probably not harmful and may add some benefit. Prolonged administration of prednisone may enhance the development of other infectious complications.

The use of extracorporeal membrane oxygenation (ECMO) has been successful in four children with cancer and PcP with intractable respiratory failure [37].

Prevention

Patients at high risk for PcP (Table 2) should be placed on chemoprophylaxis throughout the period of susceptibility. TMP-SMX is the drug of choice. For patients who cannot tolerate TMP-SMX, highly effective alternative drugs and schedules are available (Table 3). These drugs and schedules have been evaluated predominantly in patients with AIDS, but one can expect similar results in cancer patients with reasonable certainty.

Table 2 Risk Factors for *Pneumocystis* Pneumonia: Consider for Chemoprophylaxis

Lymphoproliferative malignancies
Solid tumors with intensive immunosuppressive therapy
Corticosteroid therapy
Extensive impairment of cell-mediated immunity
severe combined immunodeficiency syndrome
CD4 T-lymphocyte count $<200/\text{mm}^3$
X-linked CD40 ligand deficiency
Bone marrow transplantation
Certain solid organ transplant recipients
Severe malnutrition
Previous episode of PcP
Low-grade lymphoid malignancies treated with fludarabine and corticosteroid
Brain tumor on intensive chemotherapy, especially with corticosteroid
Wegener's granulomatosis

TMP-SMX prophylaxis for PcP has been in use for more than 25 years in hundreds of thousands of AIDS and non-AIDS patients around the world. Moreover, treatments for cancer have changed, many with increasing intensity and suppression of the immune system. One may question whether these factors may have affected the efficacy of TMP-SMX in the prevention of PcP. Is PcP prophylaxis still needed? Has the incidence of PcP in cancer changed in recent years? What are current criteria for selection of cancer patients to receive prophylaxis? A few studies provide some insight into these questions.

The 1996 study of Lyytikainen et al. [29] gives an indication of the incidence of PcP in acute lymphoblastic leukemia (ALL) patients receiving intensive

Table 3 Drugs for Chemoprophylaxis in Cancer Patients at High Risk for *Pneumocystis* Pneumonia

Drug of choice:
Trimethoprim-sulfamethoxazole daily or 3 days per week
Adults = 320–1600 mg/day
Children = 5–25 mg/kg per day
Alternative drugs:
Aerosolized pentamidine: 300 mg once per month
Atovaquone: 1500 mg daily
Dapsone: 100 mg daily or 200 mg once per week

chemotherapy without PcP prophylaxis. Of the 29 cases of ALL, PcP occurred in 7 (24%) patients. This is similar to the incidence of 20% observed in 1977 for children with ALL [38]. Poulson et al. [39] from Copenhagen, recently sought to identify ALL patients at risk for PcP. In this 2001 study, 71 children with ALL were given TMP-SMX only during the induction and consolidation phases of anticancer therapy; the drug was stopped before the maintenance phase with oral methotrexate and 6 mercaptopurine. Of the 13 cases of PcP, all but 1 occurred within the 8 months after cessation of TMP-SMX. The investigators recommended that all patients with ALL be treated with TMP-SMX throughout the course of anticancer therapy. In a study of 51 adults with acute myeloid leukemia, 1 (5.6%) of the 18 not receiving TMP-SMX during the first course of chemotherapy developed PcP, whereas none of the remainder given prophylaxis throughout the therapy had PcP [40].

In contrast to experience before 1990, reports appeared in 1991 [41] and 1994 [42] of the onset of PcP within the first month of intensive chemotherapy for ALL. Starting TMP-SMX prophylaxis after 2 to 3 weeks of chemotherapy in children with ALL at St. Jude Children's Research Hospital has been totally effective and well tolerated [43]. At the Children's Medical Center of Dallas, all children undergoing chemotherapy with or without radiotherapy receive TMP-SMX or aerosolized pentamidine prophylaxis starting at the time of diagnosis and continuing until 6 months after cancer therapy [44]. Although the risk of PcP during the first month of chemotherapy is low, it seems prudent to introduce TMP-SMX prophylaxis, if tolerated, early in the course of anticancer therapy, especially if corticosteroid drugs are a part of therapy,

Weinthal et al. [45] used aerosolized pentamidine in PcP prophylaxis in 18 children aged 6 months to 16 years with leukemia who could not tolerate TMP-SMX. No PcP episodes occurred during the 9307 observable days and 309 monthly courses of prophylaxis. Two patients experienced cough and wheezing and two had nausea after the treatments. Similar results were found in nine children studied by O'Sullivan [46]. Mustafa, et al. [44] administered 200 mg of aerosolized pentamidine once monthly to 60 children with malignancy who could not tolerate TMP-SMX. None of the patients developed PcP during 21,600 patient-days of observation. Only two patients had severe bronchospasm, and aerosolized pentamidine was discontinued because of toxicity in three patients (5%).

Consideration of the CD4 Lymphocyte Count as a Guide for Prophylaxis

The association of a profoundly low level of CD4 lymphocytes and the high risk for PcP was made in AIDS patients, and for more than a decade it has been a highly dependable guide in clinical practice for the selection of HIV-infected patients for PcP prophylaxis. The standard of practice is to monitor the CD4 lymphocyte count and percentage in HIV-infected patients, and when the cell counts drop below $200/\text{mm}^3$ (or $< 15\%$) in adults (or similar age-dependent

values in infants and children), PcP prophylaxis is begun. Unfortunately, similar studies have not been done in non-AIDS patients. Limited data suggest that low counts may identify risks for PcP in non-AIDS patients with cancer, organ transplants, and congenital immunodeficiency disorders. Case reports of a few cancer patients with PcP show low counts at the time of diagnosis. Brunvand et al. [47] and Siminski et al. [24] reported three women with breast cancer who had counts done at the time of admission with PcP. Two of the women had CD4 lymphocyte counts of $<200/\text{mm}^3$ and the third patient had a low count of $930/\text{mm}^3$. An interesting study by Fantin et al. [48] suggests the CD4 lymphocyte count may be decreased both in patients with underlying immunosuppressive diseases and in otherwise normal individuals prior to pneumonia of other causes. Of 94 patients admitted sequentially with pneumonia (26 with HIV infection and 68 HIV-negative), PcP occurred in 12 cases, all with AIDS and low CD4 lymphocyte counts. Twelve (17.6%) of the 68 patients with pneumonia but without HIV infection or other underlying disorders also had CD4 lymphocyte counts $<200/\text{mm}^3$. Nevez et al. [49] found a positive correlation of *Pneumocystis* DNA in BAL specimens from 82 non-AIDS adults and reduction in the CD4 lymphocyte counts.

Until substantial studies become available, it seems reasonable to use the CD4 lymphocyte count in clinical practice to aid in the decision to use or not use PcP prophylaxis in those cancer patients where prediction of risk is difficult or the administration of prophylactic drugs poses some hazard.

B. Solid Organ Transplantation

Overall Update

Pneumocystis complicates all types of solid organ and hematopoietic stem cell transplantations with varying degrees of involvement. Often PcP in the transplant recipient is associated with other pulmonary infections, especially cytomegalovirus (CMV) pneumonia.

A recent analysis of solid organ transplantation at a large transplant center in the US provides valuable insight into the role of PcP in the course of the transplant recipient. Gordon et al. [50] studied 1299 solid organ recipients admitted to the Cleveland Clinic Foundation between 1987 and 1996. Episodes of PcP were diagnosed by demonstration of *Pneumocystis* in the lung tissue or secretions from the airways. The use of PcP prophylaxis in the various transplant groups was not uniform during the study period. Overall, 25 (1.9%) cases of PcP occurred in the 1299 transplant recipients. One-half the cases of PcP occurred within the first 6 months of transplantation; 36% of cases occurred ≥ 1 year after transplantation, and 21% occurred ≥ 2 years posttransplantation. There was considerable variation in the transplanted organ-specific incidence rates. PcP occurred in 5% of 101 lung, 2% of 399 heart, 3.7% of 265 liver, and 0.4% of 534 kidney transplant

recipients. One-third of the PcP cases had CMV isolated from the BAL specimens. None of the PcP cases occurred while the patients were receiving prophylaxis. The authors compared these results to those of 4581 solid organ transplant patients gleaned from the literature who were not receiving prophylaxis. The overall attack rate was 4.9%; however, there was considerable variation among the organ groups (33% of 106 lung, 4.2% of 895 heart, 11% of 279 liver, and 3.8% of 3301 kidney transplants [50]).

Liver Transplantation

Hayes et al. [51] found that PcP occurred in 8 (5.2%) of 154 adult liver transplant recipients at the Royal Prince Alfred Hospital. The pneumonitis occurred from 69 to 131 (mean = 950) days after transplantation. In comparison to case-matched controls, patients with PcP had more graft rejection ($p < 0.05$), received more OKT-3 ($p < 0.05$), had lower albumin levels ($p < 0.01$), and received more prednisone than controls ($p < 0.05$). Thus, these risk factors provide some guide in the selection of cases for PcP prophylaxis. PcP was found in 9 (10%) of the 86 children with liver transplants surviving ≥ 6 weeks at the University of Nebraska Medical Center [52]. The mean time from transplantation to diagnosis of PcP was 28 weeks (range 7 to 73 weeks). In the review by Gordon et al. [50] of data from three medical centers, PcP occurred in 31 (11%) of 279 liver transplants and 7 (23%) of the 31 patients died.

TMP-SMX is the drug of choice for the treatment and prevention of PcP. For those who cannot tolerate TMP-SMX prophylaxis, several options are available, as mentioned in the discussion of cancer. Recent studies have compared TMP-SMX with sulfadoxine-pyrimethamine (Fansidar) [53]. A total of 120 liver transplant patients were randomized to receive either TMP-SMX or sulfadoxine-pyrimethamine during the first 6 months posttransplantation. The results showed these drug combinations to be similar for efficacy and safety. It is noteworthy that one-half the patients in each group had CMV infections.

Meyers, et al. [54], at Mt. Sinai School of Medicine in New York, recently evaluated atovaquone for the prevention of PcP in 28 liver transplant patients who were intolerant to TMP-SMX. With the low dose of 750 mg of atovaquone once daily over a period of 1 year, no cases of PcP occurred. Adverse effects stemmed mainly from the gastrointestinal tract.

Saukkonen et al. [55] used aerosolized pentamidine as an alternative to TMP-SMX in 18 liver transplant patients. No cases of PcP occurred, but these patients were observed for only an average period of 4.28 months.

Fishman [56] points out that TMP-SMX may effectively prevent other opportunistic infections in addition to PcP, including toxoplasmosis and community-acquired respiratory, gastrointestinal, and urinary tract infections.

Kidney Transplantation

Some data suggest an increase of PcP in renal transplant patients in the 1990s. Branton [57] found that PcP occurred in only 13 (1.1%) of 1217 patients at the University of Nijmegen before 1991, but the incidence increased to 28 (11.5%) of 243 renal transplant recipients in 1991 and afterward. No PcP prophylaxis was used and no explanation for the increase was found. When TMP-SMX prophylaxis was introduced in 1993, no cases of PcP occurred in the subsequent 140 patients.

Recent information on the incidence and potential risk factors of PcP in renal transplant recipients comes from the study of 1129 renal transplants by Lufft et al. [58]. Four immunosuppressive regimens were used and no PcP prophylaxis was given. The PcP attack rates ranged from 0.6% in patients receiving cyclosporine plus azathioprine with or without antithymocyte globulin to 14.3% in patients receiving FK 506 plus azathioprine and prednisone. The study shows the impact of specific immunosuppressive regimens on the incidence of PcP and provides a guide for selection of patients for PcP prophylaxis. Spieker et al. [59] found OKT-3 to have a profound effect in provoking PcP. Seven (11.2%) of 62 renal allograft recipients not receiving prophylaxis developed PcP.

In Basel, Reichenberger et al. [60] found that, among 71 renal transplant recipients, those receiving immunosuppression containing tacrolimus or mycophenolate mofetil had significantly higher rates of PcP and CMV infection compared to those given cyclosporine-based regimens (65 vs. 30%). This study is in contrast to others, where mycophenolate mofetil might possibly have had a protective effect for PcP. Mycophenolic acid is well known for broad antimicrobial activity. Oz and Hughes [61] showed in the experimental rat model that mycophenolate mofetil had potent anti-PcP activity. In three large, controlled clinical trials of immunosuppressive regimens in renal transplantation the cumulative results show that PcP occurred in none (0%) of the 747 patients receiving mycophenolate mofetil and in 7 of the 405 patients receiving corticosteroids or azathioprine ($p < 0.0007$) [62–64].

Several studies have indicated that CMV infection is a risk factor for PcP in renal transplant patients. In a case-controlled study, 15 renal transplant patients with PcP were compared with 95 control cases. The risk of PcP was increased in cases of CMV infection irrespective of rejection treatments [10]. Of 7 patients with PcP in Paris, 6 had CMV infection and at least one episode of graft rejection [65]. Uchida et al. [66] found 9 (5%) cases of PcP in 174 renal transplant patients, and 3 of these also had CMV infection. There is no convincing evidence to show a direct effect of the CMV virion on *Pneumocystis*.

Chemoprophylaxis with TMP-SMX effectively prevents PcP in susceptible renal transplant recipients. For patients who cannot tolerate TMP-SMX because of adverse reactions or allergy, alternative drugs are available, and some have

been studied in renal transplant recipients; however, all have been studied extensively in AIDS patients. No episode of PcP occurred in 17 renal transplant patients given 300 mg aerosolized pentamidine at Boston Deaconess Hospital [55]. Spieker et al. [59] found only 1 (1.4%) case of PcP in 71 OKT-3-treated patients receiving monthly aerosolized pentamidine, compared to 7 (11.2%) of 62 renal transplant recipients who received no prophylaxis.

Heart Transplantation

One of the most comprehensive studies of PcP in heart transplant recipients was reported by Cisneros et al. in 1998 [67]. This multicenter study of 307 heart transplant patients found that PcP occurred in 11 (3.6%) of the individuals. PcP prophylaxis was not given routinely at all centers and none of the 11 patients with PcP had received prophylaxis. The clinical features of PcP included fever in 91%, cough in 73% and tachypnea in 73% of these cases. Episodes of pneumonitis occurred from 19 to 242 days posttransplantation (median 89 days). The mortality rate was 45%. In 2001, Cardenal [68] found PcP in 5 (7.0%) of 72 heart transplant patients in Spain.

Munoz et al. [69] reported similar results. Of 138 patients transplanted before 1994, when no prophylaxis was used, 5 (3.6%) developed PcP. After TMP-SMX prophylaxis was instituted in 1994, none of the subsequent 50 heart transplant patients developed PcP.

Additional studies support the use of PcP prophylaxis in heart transplantation. Olsen et al. [70] prospectively randomized 58 cardiac transplant patients to receive TMP-SMX daily or 3 days a week or no prophylaxis. Of the 17 controls, 7 (41%) developed PcP, whereas none (0%) of the 41 individuals given TMP-SMX developed pneumonitis. When no prophylaxis was used in 152 patients (< 1 year of age) at the Loma Linda Medical Center 10 (7.0%) episodes of PcP occurred [71]. It is helpful to know that CD4 lymphocyte counts available in 5 of the patients with PcP ranged from 158 to 1358/mm³ (mean 413), values well below the normal for age. Grossi et al. [72] found the median CD4 lymphocyte count to be 211/mm³ (range 28 to 739) in 7 cases of PcP in heart transplant recipients.

Lung Transplantation

During the decade from 1987 to 1996, PcP occurred in 5 (5%) of 101 lung transplant patients at the Cleveland Clinic [50]. Protocol-driven prophylaxis required one double-strength tablet of TMP-SMX daily for 1 year. However, no patient with PcP was receiving TMP-SMX at the time of onset. The incidence rate during the first year posttransplantation was 26 per 1000 person-years and 19.6 cases per 1000 person years after the first posttransplant year. The attack

rates for PcP in earlier studies of lung and lung-heart transplants ranged from 16 to 43% [73,74,76].

C. Bone Marrow/Hematopoietic Stem Cell Transplantation

Data from analysis of 19 bone marrow transplant patients and PcP at the University of Minnesota provide a composite of clinical features [77]. Presenting symptoms include cough, dyspnea, and fever in $> 75\%$ of patients. Chest radiographs reveal bilateral infiltrates in 58% of cases, but 15% have minimal or no abnormal radiographic findings. The organism was identified in 87% of BAL specimens, but 13% of cases required a biopsy for diagnosis. Overall survival was poor, with a 63% mortality rate. Onset of PcP during the first 6 months after transplantation was associated with a higher mortality (89%), compared to 40% with onset > 6 months after transplantation ($p < 0.0001$). Recently, PcP has been reported to occur as early as 12 days after allogeneic peripheral blood stem cell transplantation [78]. Most cases of PcP occur > 2 months posttransplant. Generally, from 5 to 15% of patients develop PcP after bone marrow transplantation if no prophylaxis is given [79–81].

A recent study by Mansharamani et al. [82] provides helpful information on the use of CD4 lymphocyte count (and percentage) for assessing the risk for PcP in immunosuppressed patients without HIV infection. The median CD4 lymphocyte count was $61/\text{mm}^3$ for 22 patients with PcP, compared to $832/\text{mm}^3$ for healthy adult controls ($p = 0.0001$). Others have also reported profoundly decreased CD4 lymphocyte counts in patients with PcP and bone marrow transplantation [83].

Several drugs are now available for the prevention of PcP in bone marrow transplant recipients. TMP-SMX is the preferred drug for chemoprophylaxis. Survey of 18 bone marrow transplant centers in the United Kingdom (UK) showed that 17 (94%) of the centers routinely gave TMP-SMX to bone marrow transplant recipients [84]. During a 5-year period when 818 autologous and 1007 allogeneic bone marrow transplants were done in the UK centers, only 12 (0.65%) cases of PcP occurred. Careful evaluation of episodes of pulmonary infections with fiberoptic bronchoscopy and BAL of 174 adults undergoing bone marrow transplantation at Memorial Sloan-Kettering Cancer Center between 1997 and 1999 and receiving PcP prophylaxis revealed no cases of PcP [85].

Alternatives to TMP-SMX for prophylaxis include aerosolized pentamidine, dapsone, and atovaquone. In the year 2000, Vasconcelles et al. [86] compared prophylaxis regimens used at Brigham and Women's Hospital for bone marrow transplantation. After transplantation, 160 mg TMP-800 mg SMX orally bid three times per week; aerosolized pentamidine, 150 mg every 2 weeks or 300 mg monthly; or dapsone 100 mg orally each day was administered. Prophylaxis was continued for 1 year posttransplantation. PcP occurred in 8 (2.4%) of 327 patients

(0% of 105 receiving TMP-SMX; 9.1% of 44 on aerosolized pentamidine; and 3.2% of 31 patients receiving dapsone). Although aerosolized pentamidine was associated with fewer adverse effects, it was less effective than TMP-SMX and had an increased risk of other infections and higher mortality at 1 year posttransplantation. Another recent retrospective study by Souza et al. [81], comparing 111 allogeneic blood and bone marrow transplant patients who were given 50 mg dapsone bid three times per week, with 535 similar patients who received TMP-SMX daily. PcP occurred in 7.2% of dapsone patients and 0.37% of those on TMP-SMX. Some variables in experimental design could have explained the difference, including the selection of dapsone patients from individuals who had become intolerant to TMP-SMX. Maltezou et al. [87] found no cases of PcP among 35 children with bone marrow transplants treated with dapsone, 50 mg/m² of body surface once per week.

No cases of PcP occurred in 43 bone marrow transplant recipients given aerosolized pentamidine every 4 weeks for 6 months [88]. One-third to one-half of these patients had detectable pentamidine in the serum (7.5 to 9 ng/mL).

Atovaquone has advantages of safety and efficacy for PcP prophylaxis in bone marrow transplant patients. Colby et al. [89] conducted a prospective, randomized study in 39 autologous peripheral blood stem cell recipients given TMP-SMX or atovaquone 1500 mg per day. Drugs were administered from -5 until -1 day, discontinued from day 0 to engraftment, then resumed 3 days per week until +100 day posttransplant. No cases of PcP occurred and atovaquone was well tolerated.

D. Primary Immunodeficiency Syndromes

The early studies of McKay [90], Burke and Good [91], and Walzer et al. [92] established the clinical relationship of PcP and primary congenital immunodeficiency disorders, predominantly those with impaired cell-mediated immunity. Few medical centers have a sufficiently large number of patients with primary immunodeficiency disorders for comprehensive studies of PcP in specific immune defects. Recently, Berrington et al. [93] reported the occurrence of PcP as the presentation of illness in 10 (20%) of 50 infants with severe combined immunodeficiency (SCID) syndrome from 1992 to 1998. All of the 10 infants were successfully treated, in contrast to high mortality rates in earlier studies of PcP in SCID. In a larger series, 19 (16%) of 117 infants with SCID presented with PcP as the initial illness leading to diagnosis of the underlying disorder [94].

PcP is now recognized as a risk factor for children with the X-linked hyper-IgM syndrome, caused by mutations in CD40 ligand gene [95-97]. Khawaja et al. [98] reports the results of bone marrow transplantation in 8 boys with x-linked CD40 ligand deficiency. Of the 8 patients, 6 had PcP before transplantation. The transplantation was successful in 4 of the patients, who now have normal immune

function. Corticosteroid-suppressed rats can be protected from PcP by administration of a murine CD40 ligand/leucine-zipper protein [99].

E. Evolving Risk Groups

Advances in the treatment of several diseases now include new drugs and biological agents that suppress the immune system, especially cell-mediated immunity. Evidence is accumulating to permit speculation that PcP may be associated more frequently now and in the future with underlying diseases not heretofore thought to be significant risk factors.

PcP must now be considered in patients with connective tissue diseases receiving cytotoxic agents, corticosteroids, and other immunosuppressive agents. Godeau et al. [100] reviewed 34 cases of PcP in patients with connective tissue diseases in France. The underlying diseases were Wegener's granulomatosis, $n = 12$; systemic lupus erythematosus, $n = 6$; polyarteritis nodosa, $n = 4$; poly/dermatomyositis, $n = 5$; others, $n = 7$. At the time of diagnosis of PcP, 94% of the patients were receiving corticosteroids; 70% were taking cytotoxic drugs, and 91% were lymphopenic. The mortality rate was 32%.

Ward and Donald [101] reviewed 233 patients with PcP and connective tissue diseases admitted to California hospitals from 1983 to 1994. The frequency of PcP ranged from 89 cases per 10,000 hospitalizations per year in patients with Wegener's granulomatosis to 2 cases per 10,000 hospitalizations per year in patients with rheumatoid arthritis. In-hospital mortality was 45.7%. Compared with 5457 patients with connective tissue diseases and pneumonia due to other organisms, patients with PcP were more likely to be male, white, and younger and to have Wegener's granulomatosis, lupus erythematosus, inflammatory myopathy, or polyarteritis nodosa rather than rheumatoid arthritis.

PcP occurred in 11 (6%) of 180 patients with Wegener's granulomatosis at the National Institutes of Health in Bethesda, Maryland [102]. All of the PcP patients were lymphopenic and were receiving a corticosteroid plus a second immunosuppressive drug. Aside from PcP prophylaxis, TMP-SMX has been evaluated in a randomized, placebo-controlled study for other complications of Wegener's granulomatosis [103]. Forty-one patients on TMP-SMX had fewer respiratory tract infections, and nonrespiratory tract infections and remained in remission longer than in the 40 placebo controls. These studies support the use of PcP prophylaxis in Wegener's granulomatosis.

Reports of other disease entities are listed in Table 4, with references. In essentially all instances the occurrence of PcP is related to the immunosuppressive treatment rather than the primary disease. The use of methotrexate alone or with other drugs for rheumatoid arthritis and the use of cyclosporine and corticosteroid drugs in the treatment of ulcerative colitis are examples. At present physicians

Table 4 Evolving Risk Groups for *Pneumocystis* Pneumonia^a

Asthma (104–110)
Chronic obstructive pulmonary disease (111)
Cystic fibrosis (112, 113)
Cutaneous diseases treated with immunosuppressive drugs (114–117)
Elderly adults without predisposing disease (118, 119)
Epstein-Barr virus infections (120)
Lupus erythematosus (115, 121–129)
Pulmonary emphysema (130)
Rheumatoid arthritis (100, 101, 131–139)
Sudden infant death syndrome (140–142)
Thyroiditis (143, 144)
Ulcerative colitis (145–149)
Wegener's granulomatosis (101–103, 150–152)

^a Reports since 1990.

should give attention to the type and extent of immunosuppressive treatment of patients with these and other chronic diseases with respect to PcP.

F. *Pneumocystis* Infection in Normal People

Serological surveys of normal adults and children over the past three decades have shown that the majority of presumably normal individuals acquire antibody to *Pneumocystis* early in life, indicating subclinical infection or unrecognized clinical illness. In recent years, studies have suggested that some immunocompetent individuals without an underlying disease may become ill with PcP. Jacobs et al. [118] described a cluster of five elderly patients at the New York Hospital–Cornell Medical Center with pneumonitis and *Pneumocystis* demonstrated by BAL. Subsequently, similar cases were identified at other hospitals [153–155].

In a prospective study of 58 infants less than 6 months of age admitted to Children's Hospital in São Paulo with pneumonia, *Pneumocystis* was identified as the etiology in 3 (5.2%) of cases [156].

A 2-year prospective cohort study to determine whether clinical illness is associated with *Pneumocystis* early in life was recently reported by Vargas et al. [157]. This investigation enrolled 107 normal, healthy infants in Santiago, Chile, and followed them serially every 2 months. *Pneumocystis* DNA was identified in nasopharyngeal aspirates at the time of mild respiratory illnesses in 24 (32%) of 74 infants. Three (12.5%) of the 24 infants had apnea episodes, compared to 0 of 50 infants without *P. carinii* DNA. Seroconversion occurred in 85% of 79 infants studied during the first 20 months of life and occurred in the absence of any symptoms of disease in 21%. In serial autopsies of 534 pediatric patients

dying of causes other than cancer and HIV-infection, Vargas et al. [140] found *Pneumocystis* cysts in 4 (25%) of 16 infants who died of sudden infant death syndrome (SIDS) and in 10 (2.9%) of 342 infants who died of multiple conditions in the hospital ($p = 0.002$). Further studies of 161 SIDS infants' lungs revealed *Pneumocystis* cysts in 47 (35.1%) of 134 infants from Chile and 4 (14.8%) of 27 infants from Oxford, UK. None of the infants had *Pneumocystis* pneumonitis severe enough to account for their death. Similar studies of lungs of SIDS infants in New York and Connecticut revealed *Pneumocystis* cysts in 14% of 79 cases [141].

Further studies are needed to clearly delineate the role of *Pneumocystis* in disease of the normal immunocompetent human host. It seems reasonable to expect that the infection in the normal host may provoke at least mild to moderate respiratory illnesses.

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***Pneumocystis jiroveci* Pneumonia in AIDS Patients in the Developing World**

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I. Introduction

In the earlier phases of the AIDS pandemic, *Pneumocystis jiroveci* pneumonia was thought to occur less frequently in developing regions than in industrialized countries [1–3]. African patients, for example, were thought to develop other opportunistic infections (such as tuberculosis and bacteremia with nontyphoid *Salmonella* or pneumococci) before they could develop *Pneumocystis* pneumonia (PcP) [4,5]. More recently, studies from a number of developing countries suggest that significantly higher prevalences of PcP among HIV-infected patients exist than had previously been suspected. This article reviews reports on PcP from various developing countries and compares the epidemiological and clinical features of this disease in developing countries to that of the disease in industrialized nations.

II. *Pneumocystis* Pneumonia in Developing Countries

Wide variations in the prevalence of PcP have been reported (Table 1). These variations are due, in part, to differences in study design, including a lack of uniformity in the patient population studied (such as whether they are HIV-infected or not and whether they have respiratory symptoms or not). The variation could also be due to differences in access to health care and in the percentage

Table 1 Summary of Studies Addressing *Pneumocystis jiroveci* Pneumonia in the Developing World According to Country, Patient Characteristics, Study Design, Percent of Patients with *Pneumocystis* Pneumonia, and Outcome

Study	Years data collected	Location of study	Patient population; pneumonia only (Y/N) ^a	Study design	Diagnostic method	% of Patients w/PeP (# PeP/# total)	CD4 Count ^b (median)	Therapy; % coinfection and types; mortality in PeP cases (%)
Africa Zar et al., 2000 (22)	1998	South Africa	HIV + children; Y	Prospective clinical	IS or BAL	9.9% (15/151)	871	TMP/SMX + steroids; N/A; 47%
Graham et al., 2000 (23)	1996	Malawi	HIV + children; Y	Prospective clinical	NPA, DFA	10.7% (16/150)	N/A	TMP/SMX + steroids; N/A; 62%
Mahomed et al., 1999 (20)	1987–93	South Africa	HIV + adults; Y	Retrospective clinical	BAL, TBBx	43% (29/67)	N/A	N/A; <i>M Tb</i> 13%; N/A
Kamiya et al., 1997 (21)	1995	Malawi	Children (60% HIV +); Y	Retrospective clinical	NPA, DFA	8.3% (5/60)	N/A	TMP/SMX without steroids; N/A; 80%
Ikeogu et al., 1997 (27)	1992–93	Zimbabwe	Children (66% HIV +); N	Autopsy	Histopath, H&E, MS stains	16% (19/122) of HIV + cases	N/A	N/A; N/A; N/A
Lucas et al., 1996 (28)	1991–92	Cote d'Ivoire	HIV + children; N	Autopsy	Histopath	14% (11/78)	N/A	N/A; N/A; N/A

Jeena et al., 1996 (26)	1993-94	South Africa	HIV + children; Y (ICU deaths)	Autopsy	Histopath, H&E, MS stains	52% (16/31)	N/A	N/A; CMV 68% Adenovirus 15%; N/A TMP/SMX + steroids; <i>M.</i> <i>Tb</i> 28%; KS 15%; 10%
Malin et al., 1995 (5)	1992-93	Zimbabwe	HIV + adults, AFB smear-; Y	Prospective clinical	BAL, GMS, toluidine blue	33% (21/64)	134	
Batung- Wanayo et al., 1994 (9)	1990	Rwanda	HIV + adults AFB smear-; Y	Prospective clinical	BAL, TBBx, H&E, GMS, Mucicarmine	5% (5/111)	N/A	N/A; N/A; N/A
Lucas et al., 1993 (4)	1989-91	Cote d'Ivoire	HIV + adults autopsy; N	Clinical (natural history) then autopsy	Histopath	3% (7/247)	14 in those w/PCP	N/A; <i>Crypt</i> and <i>Nocardia</i> reported; N/A
Atzori et al., 1993 (8)	1989-91	Tanzania	HIV + adults; Y	Clinical series	IS, Toluidine blue	3% (3/83)	N/A	N/A; <i>M. Tb</i> 66%; N/A
Machiels et al., 1992 (10)	1990	Zambia	HIV + adults, AFB smear-; Y	Prospective clinical	BAL, toluidine blue	9% (4/44)	N/A	TMP/SMX, Dapsone, Fansidar; <i>M.</i> <i>Tb</i> 25%; 25%
Abouya et al., 1992 (14)	1989	Cote d'Ivoire	Adults (66% HIV+); Y	Autopsy	Histopath, H&E, MS stains	12% (10/78)	N/A	N/A; <i>M. Tb</i> 40%; N/A
Carne et al., 1991 (12)	Prior to 1990	Congo	HIV + adults; Y	Clinical series	BAL, Musto technique	11% (5/45)	N/A	TMP/SMX; N/A; 60%

(Continued)

Table 1 Continued

Study	Years data collected	Location of study	Patient population; pneumonia only (Y/N) ^a	Study design	Diagnostic method	% of Patients w/PcP (# PcP/# total)	CD4 Count ^b (median)	Therapy; % coinfection and types; mortality in PcP cases (%)
McLeod et al., 1989 (13)	1986–87	Zimbabwe	HIV + adults; Y	Clinical series	BAL, Giemsa, toluidine blue	22% (8/37)	N/A	N/A; <i>S. pneumo</i> 50%, <i>S. aureus</i> 25%, <i>M Tb</i> 25%; N/A
Elvin et al., 1989 (11)	1988	Zambia	HIV + adults; Y	Clinical	IS, indirect IFA	14% (4/27)	N/A	N/A; N/A; N/A
Ruffini et al., 2002 (24)	1998–99	South Africa	HIV + children; Y	Clinical	IS, NPA With MCA	49% (51/105) using either test	22	TMP/SMX; CMV 44%; 27%
Madhi et al., 2000 (25)	1997–98	South Africa	HIV + children; Y	Clinical	Clinical parameters only	20% (20/100)	N/A	N/A; N/A; N/A
Asia Lumbiganon et al., 2000 (40)	1989–98	Thailand	Children with AIDS	Longitudinal clinical	IS, DFA	40% (36/90)	N/A	N/A; N/A; N/A

Tansuphas- awadikul et al., 1999 (35)	1993–96	Thailand	HIV + adults; Y	Clinical history	Clinical history, PE, and CXR	4.8% (74/1553)	N/A	N/A; N/A; N/A
Oh et al., 1999 (36)	1995–98	South Korea	HIV + adults; Y	Clinical series	NS	10% (18/173)	16	TMP/SMX; 0%; N/A
Wannam- Ethee et al., 1998 (34)	Prior to 1/94	Thailand	HIV + adults; Y	Clinical series	NS	27.3% (621/2300)	Median 324	N/A; N/A; N/A; N/A
Hsiao et al., 1997 (38)	1986–96	Taiwan	Adults with AIDS; Y	Autopsy series	Histopath, H&E stain	12.5% (2/16)	All <200	TMP/SMX or pentamidine ; CMV 100% HSV 50%; N/A. N/A; 24% coinfectd crypto 25%, CMV 5%; N/A
Moot- Sikapun et al., 1996 (37)	1992–94	Thailand	HIV + adults; Y	Clinical series	IS, BAL, or TBBx, GMS, or Giemsa stain	28% (25/88)	N/A	N/A; coinfected crypto 25%, CMV 5%; N/A
Manaloto et al., 1994 (33)	1985–93	Philippines	HIV + adults; Y	Prospective clinical	Sputum for monoclonal IFA	30% (7/23)	364	N/A; None; N/A
Bhoopat et al., 1994 (39)	N/S– 1994	Thailand	HIV + children; Y	Autopsy	Histopath w/H&E and GMS stains	65% (19/29)	N/A	N/A; CMV 58% M.Tb 5%; N/A

(Continued)

Table 1 Continued

Study	Years data collected	Location of study	Patient population; pneumonia only (Y/N) ^a	Study design	Diagnostic method	% of Patients w/PcP (# PcP/# total)	CD4 Count ^b (median)	Therapy; % coinfection and mortality types; mortality in PcP cases (%)
The Americas								
Pitchenik et al., 1983 (16)	1980–82	Haiti	Haitian adults in US with AIDS; N	Retrospective clinical	Histopath or by TBBx	35% (7/20)	<100 in all	TMP/SMX; N/A; 28%
Serrano et al., 1997 (30)	1990–95	Central America	HIV + adults; Y	Retrospective clinical	NS	16% (9/55)	N/A	N/A; N/A; N/A
Rodriguez French et al., 1996 (29)	1995	Panama	HIV + adults; Y	Retrospective clinical	BAL, sputum, bronch	45% (46/55)	N/A	N/A; N/A; N/A
Mohar et al., 1992 (18)	1984–89	Mexico	HIV + adults; N	Autopsy	Histopath stained w/H&E	24% (43/177)	N/A	N/A; N/A; N/A
Wolff et al., 2001 (31)	N/S–2001	Chile	HIV + adults; N	Prospective clinical	NS	9.1 PcP events/100PY	N/A	N/A; N/A; N/A
Santoro-Lopes et al., 1998 (32)	1991–95	Brazil	HIV + adults (without AIDS); N	Natural history	NS	18% (196/1100)	N/A	N/A; N/A; N/A

Weinberg et al., 1993 (15)	1988–89	Brazil	HIV + adults; Y	Prospective clinical	IS, BAL, or TBBx; toluidine blue	43% (15/35)	N/A	TMP/SMX, pentamidine; <i>M. Tb</i> 25%, CMV 6%; 27% N/A; CMV 6%; N/A
Michalany et al., 1987 (19)	1981–85	Brazil	Adult AIDS deaths; N	Autopsy	Histopath, H&E, GMS, and Giemsa	13% (2/15)	N/A	
Moreira et al., 1993 (17)	1989–91	Brazil	Adults with AIDS; Y	Clinical series	Bronchoscopy and TBBx;	24% (27/111)	N/A	N/A; N/A; N/A

Key: Y, yes; N, no; NPA, nasopharyngeal aspirate. DFA, direct fluorescent antibody test; TBBx, transbronchial biopsy; IS, induced sputum; GMS, Gomori methenamine silver; Histopath, histopathology; H&E, hematoxylin and eosin; MS, methanamine silver; *M. Tb*, *Mycobacterium tuberculosis*; BAL, bronchoalveolar lavage; CMV, cytomegalovirus; *Crypt*, cryptococcus; TMP/SMX, trimethoprim/sulfamethoxazole, IP, inhaled pentamidine; *S. aureus*, *Staphylococcus aureus*; *S. pneumo*, *Streptococcus pneumoniae*; N/A, not available, N/S, not specified; PY, patient-years.

^aY, only those diagnosed with pneumonia; N, any HIV-related diagnosis including pneumonia.

^bNumber of cells per cubic millimeter.

of patients receiving *Pneumocystis* prophylaxis (most studies did not indicate this). Furthermore, there are variations in the sensitivity and specificity of techniques used to diagnose *Pneumocystis* infection among the studies reviewed in this report. Some studies utilize accurate methods (bronchoalveolar lavage [BAL] with appropriate staining techniques), whereas others use techniques such as nasopharyngeal aspirate (NPA), whose sensitivity is not known, as it has not been directly compared with other diagnostic methods. Nevertheless, one study suggests that NPA may be less sensitive than BAL, as NPA detected PcP in 16 of 150 children with pneumonia, but was unable to detect *Pneumocystis* infection in 24 patients in whom it was suspected clinically [23].

Another reason for the variation is differences in the degree of immunosuppression of the patient populations; most studies did not report CD4 cell counts. Thus, studies that include patients with higher CD4 cell counts would likely report a lower prevalence of PcP. This is true since there is no evidence to support the speculation that pneumocystosis may occur at an earlier stage of infection in the developing world than in industrialized countries [6]. Finally, although the natural habitat and mode of transmission, including animal-to-human transmission, are unknown, it is possible that differences of attack rates among mammals and in human exposure to various wild and domesticated mammals may also influence the prevalence of PcP. The individual studies addressing PcP in AIDS patients are reviewed according to geographic region.

III. Early Studies Carried Out During the First Decade of HIV Infection

A. Africa

The majority of clinical studies initiated and performed during the early period of the pandemic showed that *P. jiroveci* was not a common cause of HIV-infection in African adults with pneumonia. For example, one study conducted during this period showed that *Mycobacterium tuberculosis* and other bacteria were more frequent causes of pneumonia than PcP [7]. Two prospective studies, one conducted in 1990 in Rwanda [8] and another from 1989 to 1991 in Tanzania [9], reported, in fact, that PcP accounted for only 3 to 5% of cases with respiratory illness, respectively (Table 1). In addition, two other prospective studies from Zambia carried out in 1990 and 1988 [10, 11] and another from the Congo performed prior to 1990 [12] showed that PcP accounted for 9 to 14% of cases with respiratory illness. Only one study showed a higher rate of PcP than the others conducted during this period. In that study, carried out in Zimbabwe from 1986 to 1987 [13], pneumocystosis accounted for 22% of respiratory cases in that series.

In addition to the clinical trials conducted during this period, adult autopsy studies reported from Africa also reported a low prevalence of *Pneumocystis*

infection during this period. For example, one study of patients dying from any cause in Cote d'Ivoire from 1989 to 1991 [4] and another [14] of patients dying with pneumonia from the same country in 1989 showed that the prevalence of PcP was 3 and 12%, respectively.

B. The Americas

Three clinical studies from the Americas showed that the prevalence of PcP in AIDS patients during the early period of the pandemic was higher than reports from Africa. For example, one prospective Brazilian study conducted in 1988 showed that PcP caused 43% of cases of pneumonia in HIV-infected patients [15]. Also, a retrospective Haitian study, using standard diagnostic tests conducted in 1982 showed that *Pneumocystis* infections affected 35% of patients with AIDS [16]. Furthermore, a Brazilian study in 1989, also using sensitive diagnostic tests, showed that PcP was the cause of 24% of cases of pneumonia [17]. In addition, autopsy studies reported from the Americas noted a significant prevalence of PcP during this time; one Mexican study of AIDS patients who died from any cause from 1984 to 1989 [18] and another Brazilian study from 1981 to 1985 noted that PcP caused 25 and 13% of cases, respectively [19].

IV. Recent Studies Carried Out During the Second Decade

A. Africa

More recent clinical studies using sensitive methods to diagnose pneumocystosis show that *P. jiroveci* accounts for a greater percentage of cases of African adult HIV-infected patients with pneumonia than had been noted in the earlier reports. For example, one South African retrospective study using BAL and monoclonal antibody methods in HIV patients diagnosed with pneumonia from 1987 to 1993 showed that PcP accounted for 43% of episodes [20]. In another prospective study that used similar diagnostic methods in patients with pneumonia from 1992 to 1993 in Zimbabwe, 33% had PcP [4].

Studies have shown that *P. jiroveci* also causes pneumonia in a sizable proportion of HIV-infected children during this period, although the rates are more variable than the adult cases mentioned above. For example, one retrospective study from Malawi in 1995 [21] showed that PcP affected 9% of children with pneumonia, 40% of whom were HIV-uninfected. Also, two subsequent African prospective studies carried out in 1998 (South Africa) [22] and 1996 (Malawi) [23] using induced sputum methods showed that PcP only accounted for 10 and 11% of respiratory cases, respectively. However, a recent South African prospective study of hospitalized children with pneumonia conducted in 1998–1999 [24] using induced sputum techniques with direct fluorescent antibody staining showed

that PcP accounted for a much higher percentage of cases, 49%. In contrast, a study from the same group of investigators, from the same hospital, using clinical criteria only to diagnose PcP in 1997–1998, reported a lower prevalence of PcP, 20% [25].

Postmortem studies of African HIV-infected children dying either from any cause or exclusively from a respiratory illness also note a significant but variable proportion of patients with PcP. In one report, *Pneumocystis* infections accounted for 52% of patients with pulmonary infiltrates who died in an intensive care setting in South Africa during 1992–1993 [26]. Additional studies of deaths from any cause have shown that the proportion of cases is lower than series restricted to pulmonary abnormalities. For example, one study from Zimbabwe [27] showed that 16% of children who died at home from 1991 to 1992 had PcP, whereas another from Cote d'Ivoire [28] showed that 14% of deaths in HIV-infected children were due to pneumocystosis.

B. The Americas

Four South or Central American studies investigating the prevalence of PcP have been performed. Each of the prospective or retrospective descriptive studies used diagnostic methods of varying sensitivities, and reported broadly variable rates of PcP. One Panamanian study using sensitive diagnostic methods in 1995 showed that PcP occurred in 45% of HIV-infected adults with respiratory symptoms [29]. A study from the Harris County, Texas, hospital district facilities, from 1990–1995, reported that PcP was present in 45% of HIV-infected adults with respiratory symptoms [29]. That study also reported PcP was present in 16% of HIV-infected Central American (Mexico, El Salvador and Guatemala) adults with respiratory symptoms [30]. Another study of 150 HIV-infected adults from Chile reported an incidence rate of 9.1 PcP events per 100 patient-years [31]. Finally, a Brazilian study of a different design following HIV-infected adults without AIDS, from 1991–1995, reported a PcP prevalence of 19% [32].

C. Asia

In Asia, conflicting results were obtained from several studies of pneumocystosis that either did not specify the diagnostic methods used or used clinical criteria alone. In two studies conducted in Thailand [34] and the Philippines [33], PcP affected 27% of HIV-positive adults [34]. In contrast, PcP accounted for only 5% of respiratory illnesses in this population in another Thai study [35], and only 10% in a South Korean study [36].

Higher prevalence rates were found in studies using more sensitive and specific laboratory diagnostic methods. In studies of HIV-infected adults with respiratory illness from Thailand [37] and the Philippines [33], PcP was diagnosed

in 28 and 30% of cases, respectively. In another Thai study, PcP diagnosed by clinical criteria only occurred in 40% of children with AIDS followed longitudinally over a 9-year period [40].

Two autopsy studies of HIV-infected patients were conducted during this period. One reported PcP in 13% of Taiwanese adults with HIV dying with pulmonary infiltrates from 1986 to 1996 [38]. In that study, half of the patients had received treatment for presumed PcP before undergoing a postmortem examination, thereby possibly lowering the percentage of cases in which PcP was detected. The other autopsy study showed that pneumocystosis accounted for 65% of children with pulmonary infiltrates in Thailand who died before 1994 [39].

V. Trends in the Prevalence of HIV

PcP now accounts for a substantial percentage of HIV-infected patients with respiratory symptoms in the developing world. It remains speculative whether the trend toward an increase in the percentage of African cases represents a true increase in the prevalence of PcP or whether the early reports underestimated the actual prevalence.

In contrast, the reported incidence of *Pneumocystis* infections in the developed world has undergone a significant decline during the course of the epidemic. Both the use of *P. jiroveci* prophylaxis and, more recently, the use of highly active antiretroviral therapy (HAART) contributed to this decline. One U.S. study of patients with CD4 cell counts $< 100/\text{mm}^3$ showed that the incidence of PcP declined from 13 cases per 100 patient-years in 1994 to 3 cases per 100 patient-years in 1997. Because the rates of PcP prophylaxis remained constant (92%) during the study period, HAART was considered to play the key role in the reduction of PcP [41]. During this time, there was no significant change in the incidence of PcP prophylaxis, confirming that HAART was essential in reducing the incidence of PcP. In addition, a Swiss study showed a 30% reduction in the incidence of PcP between 1995 and 1997 (2.35 episodes per 100 patient-years to 0.22 episodes per 100 patient-years) [42]. Despite these therapies, PcP remained the most common AIDS-defining opportunistic infection in the US [43], accounting for 39% of cases in 1997.

VI. Comparison of Clinical Features of *Pneumocystis* Pneumonia in Developing and Industrialized Countries

Information on clinical features, radiological patterns, or laboratory abnormalities of PcP in developing countries is sparse due to the limited number of studies that describe these features (Table 2). Nevertheless, in the papers where the clinical features are reported, respiratory rates (RR) range from 30 to 80/min (Table 2).

Table 2 Summary of studies addressing *Pneumocystis jiroveci* Pneumonia in the Developing World According to Country, Clinical findings, and Radiologic Findings

Study	Clinical Features in PcP + Pts.	X-ray Findings
Africa Zar et al., 2000 (22)	RR=63, LDH=625	N/A
Graham et al., 2000 (23)	RR=80 O ₂ sat=60% T=37.8°C (mean)	Diffuse interstitial infiltrate (69%)
Mahomed et al., 1999 (20)	N/A	Diffuse infiltrate (92%)
Kamiya et al., 1997 (21)	O ₂ sat=82% RR > 70 in 60%, malnourished	Diffuse infiltrate (50%)
Malin et al., 1995 (5)	RR>40 predicted PcP (OR=11.2)	N/A
Batung-Wanayo et al., 1994 (9)	N/A	Diffuse infiltrate (40%)
Machiels et al., 1992 (10)	Diarrhea, wasting, cough, fever, dyspnea	Bilateral infiltrate (75%)
McLeod et al., 1989 (13)	Cough, weight loss, fever (80%)	Diffuse interstitial (50%), miliary (15%)
Moot-Sikapun et al., 1996 (37)	Fever, SOB, dry cough, RR>30, P _{O₂} <70	Diffuse infiltrates all
The Americas Pitchenik et al., 1983 (16)	SOB and fever in 6 of 7	Diffuse infiltrates all
Weinberg et al., 1993 (15)	55% w/mean 6.1 kg of weight loss	N/A

Key: RR, respiratory rate (mean); LDH, lactate dehydrogenase (mean); O₂sat, oxygen saturation (mean); T, temperature; N/A, not available; SOB,

In addition, the O₂ saturation percent is low in two papers in which it is reported (60 and 82%), and Po_{o2} is low in one paper in which it is recorded (70 mmHg). Together, the severity of the findings in these reports suggests that either PcP in developing countries is diagnosed in an advanced stage or that studies are biased toward selection of the most severe cases of infection.

Only a minority of the reports includes information on CD4 cell counts (Table 1), and only one of these studies specifies the CD4 cell counts of patients according to the presence of PcP (14/mm³) [4]. The mean cell counts of all patients from adult and pediatric series that report CD4 values are 871/mm³, 134/mm³, 324/mm³, 20/mm³, and 16/mm³; the corresponding PcP percentages for each of these series are: 9.9, 30, 33, 49, and 10% (Table 1). Thus, by including patients with CD4 cell counts above the range at which PcP would be expected to occur, these series underestimate the true prevalence in the population at risk

for this infection. The possibility that PcP may occur at an earlier stage of infection in developing countries than industrialized regions is not supported by one study reporting a mean CD4 cell count of $24/\text{mm}^3$ in African AIDS patients who were diagnosed with PcP while living in England [44].

VII. Coinfection with *Mycobacterium tuberculosis* and Other Pathogens

The percentage of PcP cases that involve coinfection with other pathogens is higher in developing than in industrialized countries, ranging from 20 to 70% (Table 1). *Mycobacterium tuberculosis* is the most common copathogen, present in 14 to 66% of cases (Table 1). A Mexican study showed that tuberculosis was found at postmortem examinations in 25% of patients with AIDS, compared with 6% of U.S. and 5% of Italian patients [28]. One report also noted a significant frequency of coinfection with nontuberculous pathogens: cytomegalovirus (68%), *Streptococcus pneumoniae* (50%), *Staphylococcus aureus* (25%), and *Cryptococcus neoformans* (25%). An autopsy series of 18 children from South Africa showed a high frequency of CMV coinfection, 44%, possibly reflecting the low mean CD4 cell count ($22/\text{mm}^3$) of patients and the routine use of steroids for the treatment of PcP [9].

VIII. Treatment and Outcome

No prospective, controlled treatment trial to assess response of PcP to therapy has been carried out in the developing world. Nevertheless, mortality rates have been reported using treatment with trimethoprim-sulfamethoxazole (TMP-SMX). In adult patients, the mortality rates range from 10 to 27%—similar to rates reported in the US. However, *Pneumocystis* mortality rates in children are higher in developing than in industrialized countries, ranging from 10 to 80% (Table 1). Based on the degree of oxygen exchange impairment provided in some reports, it is possible that the lower response rates may have been due to late initiation of anti-*Pneumocystis* treatment. Also, several alternate anti-*Pneumocystis* regimens to TMP-SMX, used for those not responding to or intolerant to TMP-SMX in industrialized nations, are not available in all developing countries. Additionally, a more rapid progression of HIV infection may be an additional factor contributing to mortality, since HAART was not available in developing countries at the time the studies were conducted.

IX. Summary

PcP, once considered uncommon in the developing world, is now an important AIDS-associated opportunistic infection in many developing countries. In contrast to reports from Africa carried out in the early period of the pandemic,

Pneumocystis infection now comprises a significant percentage of HIV-infected patients with respiratory symptoms in Africa and other regions in the developing world. The benefits of TMP-SMX prophylaxis demonstrated in three double-blind, placebo-controlled trials conducted in Africa are evidence of the clinical importance of PcP in the developing world. Two of these trials showed a reduction in mortality and hospital admissions in patients with advanced HIV diseases receiving TMP-SMX prophylaxis in Cote d'Ivoire [45] and South Africa [46]. A third trial, including patients in an earlier stage of HIV infection, showed a reduction in morbidity but not in mortality [47]. These trials, combined with the papers reviewed in this chapter, provide support for the WHO/UNAIDS provisional recommendations for TMP-SMX *Pneumocystis* chemoprophylaxis use for the developing world [48]. As has been witnessed in industrial countries, HIV/AIDS strategies that employ PcP prophylaxis combined with HAART will likely reduce the significant prevalence of *Pneumocystis* infections in developing countries today.

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Immunological Features of *Pneumocystis* Infection in Humans

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I. Introduction

Pneumocystis jiroveci (formerly called *Pneumocystis carinii* f.sp. *hominis*) is an important cause of pneumonia in patients with HIV, cancer, and organ transplantation as well as in other immunocompromised hosts [1]. Although research on *Pneumocystis* has been hindered by the lack of a continuous in vitro cultivation system, animal models and molecular techniques have provided a lot of information about the immunological features of this infection. The principal experimental models have included congenitally immunodeficient mice or rats, mice rendered immunodeficient by genetic manipulation, and mice or rats immunosuppressed with exogenous agents (e.g., corticosteroids, antibodies to CD4 cells) [2]. These animals also produce a ready supply of *Pneumocystis* organisms.

In contrast to the situation in animals, relatively few immunological studies of *Pneumocystis* infection have been performed in humans [3]. A major contributory factor has been the paucity of organisms available from human specimens for laboratory work. While experimental models of *Pneumocystis* pneumonia have considerable relevance to the disease in humans, there are several factors that emphasize the importance of performing immunological studies in humans. For example, it is now clear that *Pneumocystis* organisms are genetically diverse and host-specific; in order to account for these differences, it is important that immunological studies be performed using organisms that are specific for that

host [4]. It is also known that the inflammatory/immune response to *Pneumocystis* in rodent models and in humans can have both helpful and harmful effects on the host; since the underlying pathogenic mechanisms are only partially understood, it is unclear how much of the information learned from these models can be applied to patients. Some clinical problems in humans, such as recurrent episodes of *Pneumocystis* pneumonia or the high frequency adverse reactions to anti-*Pneumocystis* drugs, cannot be studied with current experimental models.

Other chapters in this book have examined specific immunological aspects of *Pneumocystis* infection and may or may not have included discussion of the human form of the disease. The present chapter focuses on the immunological features of *Pneumocystis* infection in humans and relates these findings to what is known in animal models.

II. *Pneumocystis* Antigens

A. Characterization of the Antigens

Although a number of different antigens have been identified, the most widely studied is a complex termed the “major surface glycoprotein (MSG)” or “gpA.” Biochemical studies have shown that under reducing conditions, MSG has a molecular mass ranging from 95 kDa in human-derived *Pneumocystis* to 140 kDa in *Pneumocystis* from other animal species; under nonreducing conditions, MSG migrates as a high-molecular-weight aggregate [5–8]. MSG is the most abundant surface protein, is heavily glycosylated, and plays a central role in facilitating the adherence of *Pneumocystis* to host lung cells via extracellular matrix proteins (e.g., fibronectin, vitronectin), surfactant proteins (SP)-A and SP-D, and the mannose receptor [9–16]. MSG is also highly immunogenic and has both shared and species-specific antigenic determinants [17–23]. Passive antibody administration and adoptive transfer studies in experimental animals have shown that MSG contains protective B- and T-cell epitopes [24–27]. The results of immunization studies with native or recombinant MSG have varied, depending on the antigen preparation and animal model used [28–32].

Molecular analysis, which is presented in more detail in Chapter 4 of this book, has revealed that MSG actually represents a group of related proteins encoded by a multigene family with up to 100 genes per haploid genome [32–35]. Transcription occurs at a single telomeric expression site, the “upstream conserved sequence (UCS)””; the UCS encodes a signal peptide that helps direct to the endoplasmic reticulum and Golgi apparatus for processing and transportation to the organism surface [36]. This process results in only one MSG isoform being expressed on the surface of the organism at a time. Changing the MSG gene at the UCS causes a change in the surface MSG, which results in antigenic variation; the most likely mechanism for this change is recombination [35]. Although most

of this information has been derived from rat *Pneumocystis* infection, recent evidence suggests that regulation of MSG expression in human *Pneumocystis* infection occurs in a similar manner [37].

The ability to undergo antigenic variation may serve as a way for *Pneumocystis* to avoid the host immune response. This hypothesis has been difficult to test in the normal host because *Pneumocystis* cannot be cloned, the organism number is very low, and colonization may be transient. Individual rodents and HIV patients with pneumocystosis examined at a single point in time express multiple MSG isoforms in their lungs; this diversity most likely represents MSG expression by different populations of *Pneumocystis* organisms [38–40].

Recent studies have revealed the presence of new families of *Pneumocystis* genes that encode other surface proteins of different molecular masses that are related to MSG. Of particular note is the PRT family of genes that encode kexin-like proteases, which may be involved in the postranslational processing of MSG or have an additional role in the pathogenesis of pneumocystosis [41–44]. The PRT-1 or *kex 1* gene is a multicopy gene in rats but a single copy gene in mice and humans [45]. These studies are of interest because they illustrate our increasing appreciation of the genetic diversity and complexity of the organism's cell surface structures.

Another major antigen group that has received attention migrates as a broad band of 45 to 55 kDa and as 35- to 45 kDa in rat and human *Pneumocystis*, respectively, on immunoblot [7,18]. The gene encoding a single protein (p55) of rat *Pneumocystis* has been cloned and sequenced [46–48]. The deduced primary structure of this antigen contains seven repeats of a glutamic acid-rich motif in the carboxy portion of the molecule; as seen with other organisms (e.g., *Plasmodium*), such an immunodominant molecule might serve to divert the host's immune response [3]. The 55 protein resides in the *Pneumocystis* cell wall but has not yet been biochemically purified, and its function is unknown [49]. It is highly immunogenic and shares antigenic determinants with the 35- to 45-kDa antigen of human *Pneumocystis*. Immunization with p55 provides partial protection against *Pneumocystis* pneumonia in rats [50].

Other human *Pneumocystis* antigens have been identified, but their nature and functional significance are poorly understood. Examples include a 66-kDa moiety and antigens with a molecular mass of >95 kDa [3].

B. Detection of the Antigens in Body Tissues and Fluids

Human *Pneumocystis* antigens have been analyzed in bronchoalveolar lavage fluid (BALF) and in lung autopsy specimens [7,18,19,34,39,51,52]. One BALF study, which was performed by immunoblotting with polyclonal antiserum, found antigens determined to be specific for *Pneumocystis* in the cellular pellet rather than in the supernatant [51]. The 35- to 45-kDa antigen was found more frequently

(88% of the specimens) than MSG (49% of the specimens) in HIV patients with documented pneumocystosis; both antigens were absent or detected rarely in controls. The explanation for the lower frequency of MSG in BALF specimens may possibly be related to antigenic variation. A second study, which involved small numbers of patients, reported different patterns of reactivity of monoclonal antibodies to MSG in the BALF patients with single and recurrent episodes of pneumocystosis [34]. A third BALF study, which used a monoclonal antibody, reported the presence of a 66-kDa band in *Pneumocystis* patients [52], but it is difficult to relate these results to the data in the first two reports.

As has been done with other infections (e.g., histoplasmosis, legionellosis), detection of soluble antigens in the respiratory tract, serum, or urine is a potentially attractive, noninvasive method of diagnosing *Pneumocystis* pneumonia. One report has also demonstrated the feasibility of the use of a small dose of radiolabeled monoclonal antibody to human *Pneumocystis* followed by external scanning (immunoscintigraphy) to diagnose pneumocystosis in HIV patients [53]. However, the only technique that has become commercially available for clinical use is immunofluorescence [54].

III. Humoral Immune Responses to *Pneumocystis*

There is now considerable evidence that impaired humoral immunity predisposes to the development of *Pneumocystis* pneumonia and that antibodies participate in host defenses against the organism. Pneumocystosis has been reported in B cell-deficient mice and in patients with hypogammaglobulinemia [55–58]. Several studies in animal models have shown the beneficial effects of passive immunotherapy with polyclonal or monoclonal antibodies to *Pneumocystis* organisms or antigens [24–27,59,60]. Active immunization with *Pneumocystis* conferred protection to T cell-depleted mice and appeared to be mediated by serum antibodies [61–65]. One way antibodies contribute to host defenses against *Pneumocystis* is by acting as opsonins [13,66].

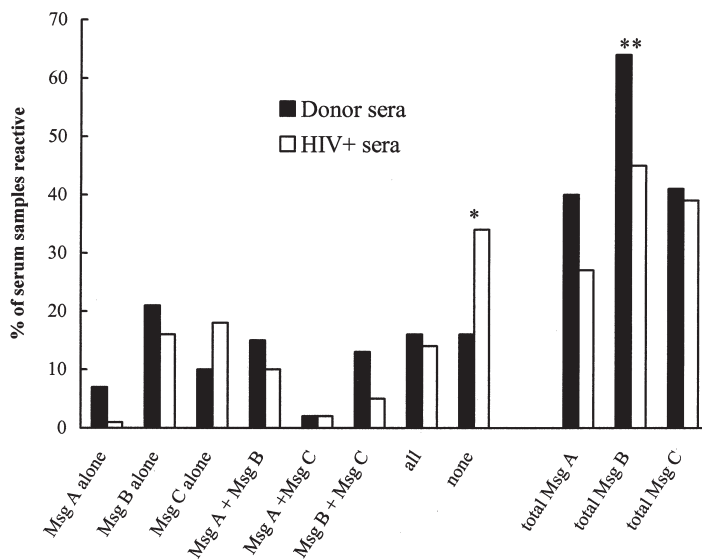
Exposure to *Pneumocystis* stimulates a serum antibody response in the host. Studies have been performed in animals to analyze this response under experimental conditions and to screen for latent infection in commercial rodent colonies [64,67–69]. Studies in humans, which were analyzed in the previous edition of this book [70], as well as those published since then [71–85] have used similar *Pneumocystis* antigen preparations: whole or fractionated organisms or antigens obtained from infected human or rodent lungs. Serological methods have included complement fixation (CF), indirect fluorescent antibody (IFA), enzyme immunoassay (EIA), and immunoblotting techniques.

Serological studies in humans have shown that exposure to *Pneumocystis* begins early in life, so that, by age 3, most children have developed antibodies to the organism [84–89]. A report that examined, by immunoblotting, 680

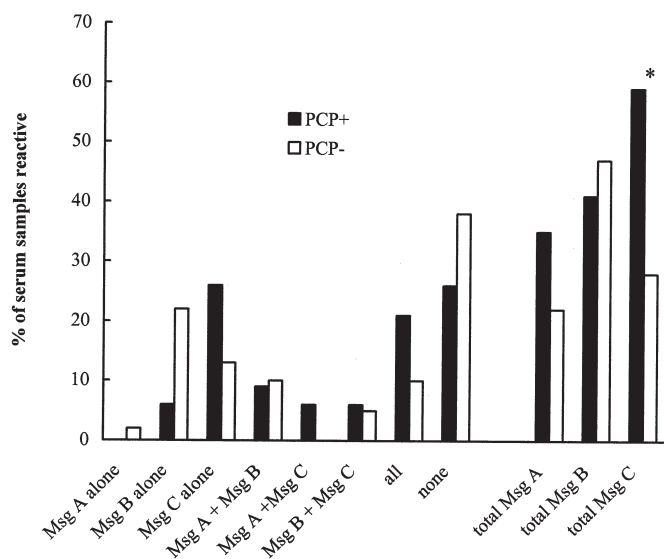
serum specimens from healthy adult subjects in several different parts of the world showed that the overall prevalence of antibodies to *Pneumocystis* was 76% [71]. The 35- to 45-kDa band was the most commonly recognized antigen (60% of the specimens), whereas the prevalence of antibodies to native MSG was only about 30%. Similar seropositivity rates of antibodies to MSG have been found in other reports, although differences have occurred among population groups and techniques used [80,81,88,90]. The frequency of antibodies to native MSG and higher-molecular-weight antigens in the large study exhibited significant geographic variation, again raising the possibility of exposure to antigenically different strains of *Pneumocystis* [71].

Despite the fact that *Pneumocystis* serology has been available for many years, its clinical application has been disappointing. In epidemiological investigations, conflicting results have been found about whether close contacts (e.g., health care workers) of *Pneumocystis* patients develop antibody responses to the organism [75,79,80,91–93]. Most serological surveys have shown that the frequency and/or level of antibodies to *Pneumocystis* among HIV patients and other immunocompromised hosts were similar to the corresponding values in healthy controls [3,70]. Thus, serology has been of little help in distinguishing between HIV or other immunosuppressed patients who have had *Pneumocystis* pneumonia, patients who have not had the disease, and controls. In addition, the frequent presence of antibodies to *Pneumocystis* in HIV patients has contributed to the belief that humoral immunity plays little role in host defenses against the organism.

On the other hand, some reports have shown that antibody responses to the organism of patients with pneumocystosis are not uniform but exhibit different patterns of reactivity. Some people experience a fall in serum antibodies before developing *Pneumocystis* pneumonia or exhibit an impaired antibody response after developing the disease [76,81,88,95–97]. Other patients develop serum antibodies to the organism with recovery from the disease [76,88,94]. In one report, active IgM and/or IgG antibody responses to the 35- to 45-kDa band by immunoblotting were found in 7 (41%) of 17 HIV patients with single episodes of pneumocystosis and in 13 (93%) of patients with recurrent episodes of the disease [88]. Active antibody responses to MSG and other *Pneumocystis* antigens were found in 12 to 29% and in 20 to 57% of the patients with single and recurrent bouts of pneumocystosis, respectively. Serum antibodies analyzed by IFA in this report demonstrated active IgM and/or IgG antibody responses were found in 24 and 71% of the patients with single and recurrent episodes of *Pneumocystis* pneumonia, respectively. Another report that used EIA showed that HIV patients who had experienced an episode of pneumocystosis had a significantly higher frequency of serum antibodies to *Pneumocystis* (66%) than did HIV patients who had never had the disease (34%) or healthy controls (35%) [90]. Almost half (43%) of the patients with *Pneumocystis* pneumonia developed a rise in IgG



a



b

Figure 1 A. The frequency of reactivity of blood donor sera and sera from human immunodeficiency virus (HIV +) patients to the MSG fragments. B. The frequency of reactivity of HIV + donors with prior PcP and those without exposure to PcP to the MSG fragments. The groups of sera were compared for reactivity to each fragment using Fisher's exact test. $p = 0.003^*$ and 0.0086^{**} when HIV – and HIV + sera were compared (A) and $p = 0.0046^*$ when PcP – and PcP + sera were compared (B). (From Ref. 85.)

antibodies over time, but the development of IgM antibodies was uncommon [72]. Taken together, these studies demonstrate that even though they are at an advanced stage of their disease, some HIV patients can mount an antibody response to *Pneumocystis*.

There is limited published information on *Pneumocystis* antibodies in BALF [3]. Some reports have shown low antibodies or impaired antibody responses [98,99] whereas others have not [100].

In 1994, the cloning and expression of human *Pneumocystis* MSG genes was reported [101]. The fact that recombinant antigens were recognized by serum antibodies offered promise that they might be helpful in serological studies. A report in 1998 identified a highly conserved region of the carboxy terminus of human *Pneumocystis* recombinant MSG that was recognized by all serum specimens tested and thus could not distinguish among HIV patients with pneumocystosis, HIV patients without the disease, and healthy controls [82].

In a more recent study, three overlapping recombinant fragments that spanned the entire length of human *Pneumocystis* MSG were generated: MSG A (MSG_{15–1119}), MSG B (MSG_{729–2282}), and MSG C (MSG_{2015–3332}) [85]. Reactivity by serum antibodies of healthy blood donors was as follows: MSG A, 40%; MSG B, 64%; and MSG C, 41% (Fig. 1A). The overall seropositivity rate to at least one of the fragments was 84%, which was much higher than the rate reported for native MSG [71,88,90]. This study also found no significant differences in recognition of the MSG fragments based on geographic origin. This result differs from our previously reported data using native MSG [71]. The reasons for these conflicting results are unclear but probably involve factors related to the use of complex antigen preparations such as lung homogenates and multiple isoforms of native MSG. It is also possible that the geographic variation involves differences in the glycosylation of MSG. Taken together, our results with recombinant MSG antigen preparations so far suggest that exposure to MSG is widespread in the general population; however, the number of epitopes recognized by serum antibodies appears to be biased towards cross-reacting epitopes.

The overall seropositivity rate among HIV patients (64%) to the recombinant MSG preparations was significantly lower than the rate among blood donors (Fig. 1A). This difference was mainly found in MSG B (45 vs. 64%). HIV patients who had a documented episode of pneumocystosis recognized MSG C significantly more frequently (59%) than HIV patients who never experienced the disease (28%) (Fig. 1B). Of note, none of nine HIV patients who went on to develop pneumocystosis after their serum specimen was drawn recognized MSG C. No significant differences were found among *Pneumocystis* and non-*Pneumocystis* patients in their reactivity to the other MSG fragments.

The results among the HIV patients here are of interest because they show how the use of different segments of an individual recombinant MSG antigen can reveal previously unrecognized differences between patient groups. A subsequent

study also found these antigen preparations was useful in measuring serum antibodies by enzyme-linked immunosorbent assay (ELISA) [85a]. These data also support a role for antibodies in host defenses against *Pneumocystis* and suggest that the epitopes that stimulate these antibodies can be localized in MSG C.

IV. Cellular Immune Responses to *Pneumocystis*

Impaired cellular immune function has long been known to predispose to the development of *Pneumocystis* pneumonia in humans. This immune impairment has been characterized by the general immune defects in the underlying diseases where pneumocystosis occurs rather than specific immune defects in the host defenses against the organism [1]. Examples of these disorders include HIV; protein-calorie malnutrition; prematurity; corticosteroids and other immunosuppressive drugs used to treat organ transplantation, cancer, and rheumatological diseases; and severe combined immunodeficiency disease (SCID) and other primary immunodeficiency disorders. Studies in animal models have shown that *Pneumocystis* pneumonia can be induced in normal mice and rats by the administration of corticosteroids or by protein-calorie malnutrition; and occurs spontaneously in athymic (nude) mice and rats and *scid/scid* mice [102–105]. Clearance of *Pneumocystis* infection is slower in neonatal mice than in adult mice; this effect is more likely to be due to factors in the lung environment rather than to T cells [106–108].

There is abundant evidence supporting the central role of CD4 cells in host defenses against *Pneumocystis* infection. The risk of pneumocystosis in HIV patients increases with a decline in the number of circulating CD4 cells, and it is recommended that *Pneumocystis* chemoprophylaxis be instituted when the CD4 count in adults falls below 200/mm³ [109,110]. Conversely, *Pneumocystis* infection may also enhance HIV replication [111]. Some reports have also found a relationship between the number of CD4 cells in peripheral blood or BALF and prognosis of pneumocystosis or the risk of adverse reactions to TMP-SMX [112,113]. Other immunosuppressed patients with CD4 counts below 200 may also be at increased risk of pneumocystosis, but this subject needs more study before recommendations about chemoprophylaxis can be made [114]. The importance of CD4 cells in the host defenses against *Pneumocystis* pneumonia has been shown by cell depletion and reconstitution experiments in rodents and by the use of knockout mice [26–29,112–117]. CD4 cells also interact with B cells and other cells via the CD40–CD40L pathway. Disruption of this pathway leads to the development of pneumocystosis in young children with the hyper IgM syndrome [118] and to impaired clearance of the organism from the lungs in mice [119].

CD8 cells accumulate in the lungs or BALF in experimental animals with pneumocystosis and have been shown to contribute to host defenses against the

organism [120–123]. CD8 cells also increase in BALF of HIV patients with *Pneumocystis* pneumonia and can be infected with HIV [124,125].

A few functional studies of cellular immune function have been performed in humans [126–130]. These reports have mainly involved analysis of proliferative and cytokine responses of peripheral blood mononuclear cells to crude *Pneumocystis* antigen preparations that were similar to those used in antibody studies. A vigorous proliferative response to native MSG and to the mitogen, concanavalin (Con) A, was found in healthy control adults, whereas the response fell in HIV patients with the decline in CD4 cells and progression of the disease (Table 1) [129]. Production of the Th1-like cytokine interferon (IFN)- γ in response to MSG followed a similar pattern; however, production of the Th2-like cytokine interleukin (IL)-4 was similar in the controls and HIV patients at all stages of their disease.

HIV patients who recovered from an episode of documented pneumocystosis and had a mean CD4 count of 60 exhibited a significantly higher proliferative response to MSG (but not to Con A) than HIV patients with advanced disease (mean CD4 count of 121) who had never had *Pneumocystis* pneumonia (Table 2). In addition, these patients exhibited a significantly higher IL-4 (but not IFN- γ) response to MSG. Overall, the data suggested that HIV patients retain a sufficient number of memory CD4 cells to mount a specific immune response to the organism, but this response was predominantly a Th2-like response [129].

Alveolar macrophages not only serve as the first line of host defense against *Pneumocystis* but also function as the major effector cell in clearing the organism from the alveoli [131,132]. Recognition and adherence of *Pneumocystis* to macro-

Table 1 Proliferative and Cytokine Responses to MSG by Human Peripheral Blood Mononuclear Cells Isolated from HIV-Infected Patients or Noninfected, Healthy Individuals

	Controls	HIV-Infected Patients		
		>500 CD4	200–500 CD4	>200 CD4
Number of Patients	12	14	13	26
MSG (cpm)	39503 $\pm$ 4920	41455 $\pm$ 8235	31667 $\pm$ 6582	20463 $\pm$ 3001 ^a
Con A (cpm)	47535 $\pm$ 8936	36693 $\pm$ 4932	36354 $\pm$ 5066	27164 $\pm$ 5283 ^a
IFN- γ (pg/mL)	2746 $\pm$ 451	2236 $\pm$ 682	2418 $\pm$ 767	934 $\pm$ 133 ^b
IL-4 (ng/mL)	1.48 $\pm$ 1.06	1.79 $\pm$ 1.1	1.64 $\pm$ 1.04	1.88 $\pm$ 1.36

^a*p* < 0.05; value is significantly less compared to healthy controls.

^b*p* < 0.001; value is significantly less compared to healthy controls.

cpm, counts per minute

Source: Ref. 129.

Table 2 Differences in Proliferative Responses and Cytokine Secretion to MSG by Peripheral Blood Mononuclear Cells^a

	Previous Episode of PcP	No Episode of PcP
Number of Patients	6	8
MSG (cpm)	28320±2240 ^b	15206±2386
Con A (cpm)	25847±4712	26381±4570
IFN- γ (pg/mL)	952±129	916±137
IL-4 (ng/mL)	2.60±0.34 ^b	0.75±0.65

^aCells isolated from CDC class C₃ patients (AIDS-defining illness, <200 CD4 cells) comparing those patients who had previously had an episode of PcP to those that had not had an episode.

^b $p < 0.01$; value is significantly greater following comparison within the the class C₃ patients.

Source: Ref. 129.

phages occur by several pathways. Important components of this interaction include *Pneumocystis* MSG and β -glucan, extracellular matrix and surfactant proteins, and host mannose and Fc receptors [9–16]. While *Pneumocystis* can activate macrophages in the absence of T cells, these macrophages require the presence of CD4 cells to control the infection in experimental models [133,134]. Another factor needed to clear the organism from the lungs is urokinase-type plasminogen activator, a protease that participates in the recruitment of inflammatory cells [135]. *Pneumocystis* encodes an enolase that can activate plasminogen; perhaps this function of the organism could serve to down regulate or inhibit the host plasminogen activator [136].

Studies of human alveolar macrophages have often used rat-derived *Pneumocystis* organisms. These macrophages can ingest, degrade, and kill *Pneumocystis* proinflammatory cytokines, eicosanoids, and reactive oxidants [13, 137–140]. Nitric acid is also released by macrophages, but there have been conflicting reports about its role in the host defenses against *Pneumocystis* [141–143]. HIV not only impairs the mannose receptor – mediated binding and phagocytosis of the organism but also changes the cytokine response [144–145]. Other studies have shown that *Pneumocystis* itself can decrease uptake by increasing shedding of the mannose receptor [146]. Administration of a soluble mannose receptor immunoadhesion enhances uptake of the organism [147].

Knowledge of the of the role of specific cytokines in *Pneumocystis* infection has mainly come from studies in animal models. Of the proinflammatory cytokines, tumor necrosis factor (TNF)- α and IL-1 have been shown to play an important role in host defenses against *Pneumocystis*, especially early in the course of the infection [148–152]. IFN- γ and GM-CSF also contribute to these host de-

fenses through activation of macrophages or with TNF- α (154–158). The effects of IFN- γ on the host immune/inflammatory response are complex. In one report, deletion of IFN- γ or TNF- α receptor genes did not adversely affect clearance of *Pneumocystis* infection, but deletion of receptor genes for both cytokines resulted in severe disease [159]. Some cytokines (e.g., IL-6) are produced in response to *Pneumocystis* infection, but their role in host defenses is unclear [160]; other cytokines (e.g., IL-10) modulate the host inflammatory response [161,162]; still other cytokines (e.g., IL-4, G-CSF) have no identifiable role in host defenses against the organism [64,163].

Little is known about the role of other host cells in defense against *Pneumocystis*. One study reported that *Pneumocystis* activates natural killer (NK) cells in conjunction with macrophages as described above [164]. Since pneumocystosis has occurred in people with HIV infection or other immunodeficiencies who had decreased numbers or function of NK cells, a role for NK cells in host defenses was suggested [165–168]. Another report found that neutrophils from patients with *Pneumocystis* pneumonia had a decreased respiratory burst compared with cells of healthy control subjects [179].

V. Complex Effects of the Host Immune/Inflammatory Response

Over the past decade, there has been increasing evidence that the immune response to *Pneumocystis* can have deleterious as well as beneficial effects on the host [4]. It is also clear that these effects are complex and depend, to some extent, on the animal or human host being studied. *Scid/scid* mice with pneumocystosis display few alterations in lung histology, cytokines, or surfactant system until late in the course of the disease [103–105,170,171]. Immune reconstitution, which has been accomplished by administration of splenocytes or bone marrow cells, results in marked inflammatory changes, rise in proinflammatory cytokines and chemokines, decreased compliance, and impaired oxygenation [170–174]. Other investigators have found that the adoptive transfer of CD4 cells also leads to this early hyperinflammatory response and have implicated CD4 cells expressing the CD25⁺ phenotype as the responsible cell [26–30,175]. Among the factors that can ameliorate this response are hyperimmune serum, CD8 cells, and CD4 cells expressing the CD25⁺ phenotype.

Normal mice depleted of CD4 cells and exposed to *Pneumocystis* develop pneumocystosis and influx of CD8 cells into the lungs [117,121,122]. Concomitantly, there are increased numbers of lung neutrophils, altered alveolar-capillary permeability, surfactant dysfunction, and impaired respiration [171–174]. However, when CD4-depleted mice are also depleted of CD8 cells, these changes are improved.

The pneumocystosis that develops in corticosteroid-treated rats is characterized by few inflammatory changes at the microscopic level [28,30]. A recently developed rat model of pneumocystosis induced by CD4-cell depletion also showed little inflammation [176]. Studies have found either no changes in cytokines or elevated proinflammatory cytokines in the lungs but not in peripheral blood [177]. Adoptive transfer experiments in rats differ from studies in mice in that the cells are cultured with MSG for 4 days before being injected into the recipient animals. Adoptive transfer of sensitized CD4 cells results in a early hyperinflammatory response that is similar to that found *scid/scid* mice and can be ameliorated by the addition of CD8 cells [28,29]. More recent studies have shown that the adoptive transfer of large numbers of sensitized splenocytes is also associated a hyperinflammatory response and elevated proinflammatory cytokines [178].

The underlying immune status of the host and ability to mount an immune/inflammatory response affect the clinical picture of pneumocystosis in humans. *Pneumocystis* pneumonia that develops in HIV patients is characterized by a high organism burden, mild to moderate symptoms, subacute course, and moderate mortality [1]. HIV patients who have recovered from pneumocystosis and respond to HAART with a significant increase in CD4 cell count may develop pulmonary infiltrates respiratory impairment; this problem has been termed “immune reconstitution disease” [179]. *Pneumocystis* pneumonia in non-HIV patients, which often begins after corticosteroids or other immunosuppressive drugs have been tapered, is characterized by low organism number, severe respiratory impairment, acute course, and high mortality [1].

More specific evidence supporting the contribution of the immune/inflammatory response to lung injury in HIV patients with *Pneumocystis* pneumonia comes from studies that correlated increased neutrophils and IL-8 levels in BALF more severe disease and worse outcome [180–186]. IL-8 is a potent chemoattractant and activator, and its interaction with *Pneumocystis* is mediated by MSG [187,188]. Other reports have noted alterations in TNF- α , IL-1, eicosanoids, and other inflammatory mediators, but their relationship to lung damage is not clear [189–195]. Increased levels of proinflammatory cytokines has been reported in BALF of HIV and non-HIV patients with pneumocystosis, yet these patients also have increased anti-inflammatory cytokines (e.g., soluble TNF receptors, IL-1 receptor antagonist) in peripheral blood [196,197].

HIV patients with *Pneumocystis* pneumonia who are treated with antimicrobial drugs often exhibit a deterioration in respiratory status soon after beginning therapy. The prompt administration of corticosteroids prevents or lessens this problem and improves survival [198]. One suggested mechanism for the beneficial effects of steroids involves their anti-inflammatory properties. Another possible explanation is the effects of steroids on the surfactant system. Studies in

animal models and humans have shown that pneumocystosis is characterized by a fall in surfactant phospholipids, a decline in SP-B and SP-C, and a rise in SP-A and SP-D [14,199–205]. These changes are at least partly due to suppression of phospholipid mediated by MSG [206,207]. Corticosteroids have lowered cytokine production in peripheral blood, but their effects on inflammatory mediators and surfactant components in BALF have been inconsistent [183,190,191, 195–197,208].

VI. Conclusion

Immunological studies of *Pneumocystis* infection in humans have been significantly limited by the lack of a reliable in vitro culture system, an adequate supply of organisms, and standardized reagents. Most of our knowledge has been obtained from animal models. Yet in light of increasing knowledge of the genetic diversity and host specificity of *Pneumocystis* infection, it cannot be assumed that what has been learned in experimental models can be directly applied to humans. It is also clear that the immune response/inflammation can have deleterious as well as beneficial effects on the host. Much more research is needed about the host immune response to human *Pneumocystis* infection in order to design new diagnostic, therapeutic, and prophylactic strategies.

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19

Molecular Typing and Epidemiological Insights

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I. Introduction

Molecular biological applications such as polymerase chain reaction (PCR), DNA sequencing, and various fragment analysis methods have had a tremendous impact on our understanding of the biology of the different *Pneumocystis* species and the epidemiology of *Pneumocystis* pneumonia in humans. For example, in the definitive textbook on clinical parasitology used in the 1970s, prior to the molecular revolution, PcP was thought to be caused by the protozoan *Pneumocystis carinii*. The reservoir was considered to be various nonhuman hosts, including rodents such as rats and mice, which were “*presumed to be carriers of the organism*” [1]. Infections in humans were known chiefly from a relatively small number of cases, primarily in premature and malnourished infants. With the advent of the AIDS epidemic in the early 1980s, the disease emerged as an important public health problem. Infections in HIV-positive individuals occurred in over 60% of all patients and were associated with a case fatality rate of close to 25% [2–4]. Disease was thought to be the result of activated latent infections, which were acquired very early in life.

DNA-based methods for molecular diagnostics, taxonomy, phylogenetic analysis, and strain subtyping have resulted in significant changes in our view

of this disease and its transmission in humans [5–7]. This chapter is devoted to a discussion of the various DNA-based techniques and approaches that have been and/or can be applied for the study of *Pneumocystis jiroveci*, the causative agent of disease in humans, and what has been revealed about the natural history and epidemiology of the disease in humans through the use of these methods.

II. Typing Methodologies

A. Genes Examined

Approximately 14 or more unique gene loci have been evaluated thus far for various typing applications (Table 1). With the exception of the internal transcribed spacer regions I and II of the nuclear ribosomal RNA array (ITS), a very modest level of polymorphism has been reported in *P. jiroveci* patient samples from around the world. Nevertheless, several of these genes have been used

Table 1 Partial List of Genes Evaluated for Various General Applications

Gene locus	General use	Ref.
Nuclear		
Beta-tubulin (β -tub)	Diagnostic, subtyping	92, 93
Dihydropteroate synthase (DHPS)	Subtyping	56, 59, 60
Dihydrofolate reductase (DHFR)	Subtyping	53, 54
5-Enolpyruvyl shikimate phosphate (EPSP)	Subtyping	94
Heat shock protein-70 (Phsb 1)	Diagnostic	90, 91
Manganese-dependent superoxide dismutase (SODA)	Genetic diversity/ phylogenetics	96
Major surface glycoprotein (MSG)	Diagnostic, subtyping	86–88
Nuclear ribosomal RNA		
5.8S (5.8S rRNA)	Subtyping, genetic diversity/phylogenetics	10, 37, 95
28S (28S rRNA)	Genetic diversity/ phylogenetics	97
Internal transcribed spacers (ITS)	Subtyping	10, 37, 40
Thymidylate synthase (TS)	Diagnostic	10, 95, 136
Mitochondrial		
Cytochrome B (Mt CytB)	Subtyping	35, 36
Large subunit ribosomal RNA (Mt LSU rRNA)	Diagnostic, subtyping	8, 10, 95
Small subunit ribosomal RNA (Mt SSU rRNA)	Diagnostic	32, 33

Table 2 Selected Gene Loci and the Corresponding Specific Clinical or Epidemiological Applications

Gene locus	Specific application	Ref.
DHPS	Transmission, clinical severity, antimicrobial resistance studies	16, 56, 57, 60–62, 64
ITS	Transmission, clinical severity, antimicrobial resistance studies, diagnostic	31, 37, 42, 44, 114
Mt CytB	Antimicrobial resistance studies	35, 36
Mt LSU rRNA	Transmission, carriage versus cause of disease, diagnostic	8, 9, 12, 15–17, 48
MSG	Carriage versus cause of disease, diagnostic	87
Phsb1	Viability	90, 91

very effectively for addressing specific questions of clinical or public health importance, including diagnostics, transmission patterns, severity of infection, and the failure of treatment and/or prophylaxis (Table 2). Uniform DNA oligonucleotide primers have been developed for these loci, allowing consistent PCR amplification of gene fragments from clinical and environmental specimens (Table 3).

mtLSU rRNA

The first gene to be identified and evaluated for diagnostic use was the mitochondrial large subunit ribosomal RNA (mtLSU rRNA) locus [8]. Both single-stage and nested primer sets have been developed (see Table 3) and work effectively and consistently on a diverse range of clinical and environmental samples, with a very high degree of sensitivity and specificity for *Pneumocystis* spp. [9–11], and with cross reactions to other fungal taxa virtually nonexistent. These primer sets do not allow distinction of *P. jiroveci* from *Pneumocystis* spp. from other mammals based solely on PCR products evaluated on horizontal agarose gels; however, when used in conjunction with direct DNA sequencing or fragment analysis methods such as single-stranded confirmation polymorphism (SSCP) (see below), the amplified DNA sequences are highly diagnostic at the species level and can distinguish *Pneumocystis* species from different mammalian hosts. In addition to diagnostic use, this locus has also been utilized for various subtyping applications, including analysis of the cause of recurrence [12,13], the possibility of person-to-person transmission [7,14,15], the evaluation of latency and reactivation vs. recent active transmission [16], and the investigation of subclinical carriage and/or potential linkage to other diseases [17–22]. In addition to being

Table 3 Universal Primer Sets for PCR Amplification of *Pneumocystis* Gene Loci Commonly Used for Epidemiological Applications

Gene locus	Primer sequence	Ref.
Beta-tubulin		
Forward	5'-TCA TTA GGT GGT GGA ACG GG-3'	92
Reverse	5'-ATC ACC ATA TCC TGG ATC CG-3'	92
DHFR		
RDHFR 11	5'-GTT GCA CTT ACA ACT TCT TAT GG-3'	55
RDFHR 12	5'-TAG ATC CAG AGA TTC ATT TCG AG-3'	55
DHPS (nested)		
First round	5'-CCT GGT ATT AAA CCA GTT TTG CC-3'	16
	5'-CAA TTT AAT AAA TTT CTT TCC AAA TAG CAT C-3'	56
Second round	5'-GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3'	56
	5'-GGA ACT TTC AAC TTG GCA ACC AC-3'	56
ITS (nested)		
First round	5'-AAG TTG ATC AAA TTT GGT C-3'	10
	5'-CTC GGA CGA GGA TCC TCG CC-3'	10
Second round	5'-CGT AGG TGA ACC TGC GGA AAG GAT C-3'	10
	5'-GTT CAG CGG GTG ATC CTG CCT G-3'	10
Mt LSU rRNA (nested)		
First round	5'-GTG TAC GTT GCA AAG TAC TC-3'	8
	5'-GAT GGC TGT TTC CAA GCC CA-3'	8
Second round	5'-GTG AAA TAC AAA TCG GAC TAG G-3'	33
	5'-TCA CTT AAT ATT AAT TGG GGA GC-3'	33
Nuclear 26S rRNA		
Forward	5'-GAA GAA ATT CAA CCA AGC-3'	92
Reverse	5'-ATT TGG CTA CCT TAA GAG-3'	97

highly sensitive and specific, the PCR assay for this locus is very reproducible and free from technical difficulties. This assay has been used successfully to demonstrate *Pneumocystis* DNA in rural outdoor settings, including apple orchards [23], pond water [24], and dust samples from homes [25], in hospital rooms and hallways [26,27], and in various clinical specimens including induced sputum, bronchoalveolar lavage specimens, oral washes, tracheal aspirates, nasopharyngeal swabs, and tissue biopsies [15,16,21,28–31].

mtSSU rRNA

A second mitochondrial gene, which has been utilized much less frequently but nevertheless has potential value for diagnostics and possibly for typing applications, is the mitochondrial small subunit ribosomal RNA (mtSSU rRNA) locus. Hunter and Wakefield [32] have described a PCR assay for this gene and shown its usefulness for distinguishing *Pneumocystis* species from different mammalian hosts (see Table 3). This locus has also been examined in *P. jiroveci* samples from pre-AIDS-era patients, where no differences were observed in comparison to the sequence seen in HIV + patients [33]. Information on the absolute sensitivity and specificity of this assay is scarce; however, sidewise comparisons between this PCR and the nested PCR at the mtLSU rRNA locus (Table 3) shows that the nested mtLSU rRNA PCR assay is much more sensitive than the single-stage assay for the mtSSU rRNA locus [22]. The relative lack of polymorphism observed at this locus to date suggests that its usefulness for subtyping applications in *P. jiroveci* is probably limited.

Mitochondrial Cytochrome b Locus

A third and final mitochondrial locus that has been used successfully for amplifying *P. jiroveci* DNA primarily from clinical specimens is the cytochrome *b* locus. The protein encoded by this gene is a key enzyme in the electron transport system and the target of the antiparasitic agent atovaquone, which is used as a second-line drug for the prophylaxis and treatment of *Pneumocystis* pneumonia [34]. A PCR protocol has been developed for amplification of this locus and subsequent analysis of genetic polymorphism [35]. DNA sequence analysis of *P. jiroveci* samples from patients with *Pneumocystis* pneumonia revealed nonsilent mutations in the Q₀ region of the gene, one of two target binding sites of cytochrome *b* coenzyme Q for the drug atovaquone. Mutations in this region have been associated with atovaquone exposure in patients with AIDS, suggesting the possible emergence of resistance to this drug [36]. Polymorphism at this locus, as with most of the other loci evaluated to date, is generally limited, indicating that while it may yield valuable clinical information, its usefulness for other subtyping applications will be very modest.

In addition to the three mitochondrial genes listed above, there are at least 11 nuclear loci that have been examined in multiple studies and have been shown to have value for diagnostic and/or subtyping activities. The single locus that has been utilized far more than any other to date is the internal transcribed spacer regions of the nuclear ribosomal RNA array (ITS1 and ITS2).

ITS

The ITS region (1 and 2, collectively) demonstrates far more polymorphism than any other *Pneumocystis* genes examined. In *P. jiroveci*, a total of at least 21 unique ITS1 and 29 ITS2 genotypes have been described to date [37–40]. The PCR assay for this locus (see Table 3) is highly reproducible and has been utilized widely for molecular diagnostic purposes. Additionally, it has been shown to have very high sensitivity and specificity for *Pneumocystis* spp. [10,11]. It should be noted, however, that this specificity is at the genus level. The PCR assay alone does not distinguish, for instance, between *P. jiroveci* from humans and *P. carinii* from rats, which in fact is typically used as a positive control in PCR diagnostic assays. If the amplified fragments are further analyzed, however, either by direct DNA sequencing or by a fragment analysis method such as SSCP, as with the mtLSU rRNA locus, *P. jiroveci* can be readily distinguished from other *Pneumocystis* spp. The ITS locus has proven useful for diagnostic purposes. It has also been utilized for various molecular epidemiological applications, including analysis of cluster cases, person-to-person transmission, potential *Pneumocystis* Pneumonia relapses, phylogenetic studies, and disease severity [15,31,37,41–48]. There are some concerns and constraints about the PCR assay and its appropriate use for different applications. These concerns relate primarily to the rapid evolutionary rate of the locus and to technical issues involving sequence-induced *Taq* polymerase error, which can introduce sequence artifacts into the resulting PCR-amplified gene fragments. These issues are discussed in greater depth in a later section of this chapter.

Drug Target Loci

Two genes that have been of great interest in recent years due to their potential implication in the emergence of antimicrobial resistance to anti-*Pneumocystis* drugs are the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) loci. Both genes encode enzymes that have key functions in folic acid metabolism. The combination of trimethoprim (TMP) and sulfamethoxazole (SMZ) (i.e., Bactrim, Septra, co-trimoxazole) is the primary antimicrobial agent used for both the prophylaxis and treatment of *Pneumocystis* Pneumonia. These two drugs target, respectively, DHFR and DHPS; however, clinical studies suggest that it is the sulfonamide component that is solely responsible for the anti-*Pneumocystis* activity of the combination drug [49]. The consistent long-term

use of TMP-SMZ both for prophylaxis and treatment has no doubt imposed great selection pressure on *P. jiroveci*, as it has done for several of the important bacterial pathogens in HIV-infected individuals [50,51].

PCR assays have been developed for both the DHFR and DHPS loci (Table 3). The original DHFR PCR primers were designed based on the rat *P. carinii* DHFR gene sequence [52] and did not amplify consistently in human (*P. jiroveci*) samples. More recently, however, primer sets have been developed that allow amplification of *P. jiroveci* clinical specimens using a nested PCR [53]. These primers are reported to work consistently and have been used to evaluate the potential emergence of TMP-SMZ resistance in *P. jiroveci* [53,54]. Additionally, a real-time quantitative touchdown PCR assay has also been developed [55]. This assay has been reported to be rapid, sensitive, and reproducible and can be used potentially for both *in vitro* and *in vivo* applications.

A nested PCR for the DHPS locus has also been described, originally by Lane et al. [56], with modifications made by other investigators [16,57]. The nested DHPS PCR works very consistently and has been used in laboratories around the world to study failures in the prophylaxis and treatment of *Pneumocystis* pneumonia and the suspected emergence of antimicrobial resistance to sulfonamide antibiotics [53,58–65]. In all of these studies, several consistent observations have been made with respect to gene sequence polymorphism: [1] variation is primarily limited to two nucleotide positions, 165 and 171; [2] the mutations observed are nonsilent, resulting in amino acid changes (Thr > Ala at position 55 and Pro > Ser at position 57); [3] these mutations are located in a functional domain of the corresponding enzyme, which is presumed to be involved in drug binding; and [4] homologous mutations in other microorganisms are associated with clinical resistance to sulfonamide antimicrobials [51,56]. Additionally, the occurrence of these mutations in *P. jiroveci* has been associated with sulfa exposure [53,61,62,64], treatment failure [59,63], prophylaxis failure [51,57], and reduced patient survival [60]. While numerous clinical studies demonstrate that co-trimoxazole is still the best agent for both the treatment and prophylaxis of *Pneumocystis* Pneumonia [64,66–69] when it can be tolerated, these observations nevertheless suggest that sulfa resistance is in the process of emerging in *P. jiroveci*; consequently, they support the need for active surveillance and vigilance.

Major Surface Glycoprotein

The major surface glycoprotein (MSG) is the primary protein antigen on the surface membrane of all *Pneumocystis* species examined to date, where it has been shown to function in the adherence of organisms to alveolar epithelial cells [70–77]. The observation of multiple MSG cDNAs led to the discovery that the protein is encoded by a multicopy gene family [75,78–81]. By switching the

expressed MSG, the organism can change surface antigens, which then presumably allows it to avoid the host immune response [75,82–84]. Genome organization and expression has recently been reviewed [75,83,85]. Both a standard diagnostic PCR [86] and a real-time quantitative touchdown (QTD) PCR [87] have been developed for this multilocus gene (Table 3). Preliminary studies with the QTD PCR suggest that it may have a use in establishing the difference in clinical *Pneumocystis* Pneumonia and *Pneumocystis* carriage [87]. The unique gene locus that mediates expression of the single active MSG has further been utilized for subtyping applications. A PCR has been developed for this locus, which allows amplification of a tandem repeat that is contained within the upstream conserved sequence (UCS) of this locus [88]. A region of 10-nucleotide tandem repeats is contained within the single intron of the UCS. PCR amplification of this site reveals strain-specific polymorphism that can be used in a simple and rapid method for subtyping and subsequent epidemiological studies.

Heat-Shock Protein 70

Identification and characterization of the *Pneumocystis* HSP70 gene family [89] has allowed the development of a very useful PCR assay. The assay, which was originally developed for *P. carinii* [90] and later adapted for *P. jiroveci* [91], is a reverse transcriptase (RT)-PCR-based viability assay that targets the HSP70 mature mRNA transcript. The primers were designed so that they span the boundaries of the third and fifth introns of the *Pneumocystis* heat-shock protein 70B (Phsb 1) gene locus so that no amplification product is possible from the DNA template but only from the mature mRNA. Since mRNA decays rapidly upon death of the organism, this PCR can be used to determine whether *Pneumocystis* organisms in a clinical or environmental sample are alive or dead. This assay will have very important applications for both epidemiological and clinical studies, such as efforts aimed at assessing response over the course of clinical treatment with specific antimicrobial agents.

Other Loci

There are at least six other genes for which PCR assays have been developed, allowing subsequent amplification of DNA fragments from *P. jiroveci*. These include thymidylate synthase, an intron of the beta-tubulin gene, the EPSP synthase domain of the multifunctional *aro* gene, the nuclear 26 rRNA and 5.8S rRNA genes, and the SODA (manganese-dependent superoxide dismutase) locus (Table 3). These genes have been evaluated for various purposes, including diagnostic [9,10] and epidemiological applications [9,33,92], and for the evaluation of genetic diversity [93–96]. The PCR assays for each of these have been reported to be reliable and efficient. With the exception of the beta-tubulin and nuclear 26S rRNA gene fragments, which have been incorporated into a multilocus SSCP

rapid typing method [92,97,98], their general use has been restricted more to diagnostics and broad evaluation of genetic diversity rather than for subtyping applications (see Tables 1 and 2), primarily because of the limited polymorphism observed to date in *P. jiroveci* clinical samples.

B. Methods Employed

A variety of methods have been employed for evaluating genetic diversity between and within *Pneumocystis* species.

Karyotype Analysis

One of the first molecular methods applied for subtyping *Pneumocystis* was karyotype analysis. Using this approach, it was possible to demonstrate the existence of different *Pneumocystis* special forms (now regarded as species) in numerous different mammals, including rats, mice, ferrets, and humans [99–103]. This method involves the isolation of large numbers of organisms from infected tissues and direct observation of chromosomal bands in agarose gel, separated by pulsed-field gel electrophoresis (PFGE). The genome of *P. carinii* from rats has been shown to contain 13 to 15 linear chromosomes that range in size from 300 to 700 kb, with approximately eight different karyotypes observed to date [104]. Currently, it is not clear whether these karyotype forms represent inter- or intraspecific variation. At least two of the karyotype forms have been designated as unique *special forms*—f. sp. *carinii* and f. sp. *ratti*, species—and in all likelihood will be given individual species status in the near future. Karyotype analysis has been very useful in learning important information about *Pneumocystis* genome size and general chromosomal organization; however, the application of this technique to subtyping and subsequent clinical and epidemiological studies with *Pneumocystis* Pneumonia in humans is very limited due primarily to the requirement of large numbers of organisms for analysis.

Multilocus Enzyme Electrophoresis

Another typing method, which is used very broadly and has also been applied to studies with *Pneumocystis*, is multilocus enzyme electrophoresis (MLEE). This method involves the separation of enzyme preparations in nondenaturing gels and subsequent detection of specific alleles that migrate at different rates. Qualitative and quantitative allelic differences can be used for subtyping organisms and conducting population genetic analysis. This approach has been used with *Pneumocystis* spp. to demonstrate genetic diversity and to establish the lack of gene flow between *Pneumocystis* spp. from different mammalian hosts [105–107]. As with karyotype analysis, MLEE has been very useful for evaluating diversity among the related *Pneumocystis* spp. from different mammal hosts;

however, the clinical and epidemiological applications have been very limited due to the lack of intraspecific polymorphism that can be detected using this approach.

Sequence-Based Typing

The remaining methods for typing rely on DNA sequence data or information directly related to sequence polymorphism. These methods have therefore been of much greater value for detecting intraspecific variation from which epidemiological inferences can be drawn. One of the early DNA fragment-based methods to be developed was called type-specific oligonucleotide typing (TSO) [108]. This approach was based on polymorphism observed at the ITS1 and ITS2 loci and allowed identification of two different ITS1 types and three different ITS2 types. Mixed infections, however, could not be evaluated using this approach, if the coinfecting strains were of different ITS1 genotypes. While this method is quick and inexpensive to perform, the obvious limitation is the fact that it fails to recognize all of the genotypes present at the ITS region, which most recently has been calculated to be at least 21 unique ITS1 and 29 ITS2 genotypes [40]. Consequently the method is no longer used.

Direct DNA Sequencing

The subtyping approach that is probably the most commonly utilized is direct DNA sequencing. Because sequencing provides the maximum amount of genetic information that can be obtained for any particular DNA fragment, this method is the most sensitive approach for detecting and characterizing qualitative differences. Downsides to this method include its cost and the fact that it is labor-intensive, relative to some of the other methods discussed below, which detect differences derived from the primary DNA sequence. Nevertheless, sequencing is the most thorough way to investigate genetic variation and is therefore the “gold standard” for molecular epidemiological investigations.

DNA sequencing has been used for investigating numerous questions of great clinical and epidemiological importance, including the emergence of drug resistance [53,56–65], clinical severity [31,60], case cluster investigations [15, 44,109], subclinical carriage [20,22], transmission patterns [14,16,48,109,110], recurrent infections [12,13,42,43], and genetic diversity [37,41,111,112]. In this approach, the DNA fragments are amplified by PCR and then either sequenced directly or cloned into plasmids for sequencing. Direct sequencing is usually sufficient to allow easy determination of the genotype. Mixed infections can be observed as multiple signal peaks appearing on electropherograms using automated DNA sequencing technology. Depending on the numbers of potential genotypes at a given locus and the subsequent complexity of the pattern observed for various combinations of mixed infections, it is sometimes necessary to clone the

PCR product first, before sequencing. In most cases, four to five clones are sequenced, from which the genotypes present can be resolved. Investigators using direct sequencing for genotyping *P. jiroveci* strains have reported coinfections with multiple strains to occur at frequencies ranging from 15 to 75%, depending on the patient population and the gene loci evaluated.

Single-Strand Conformation Polymorphism

The second most common method for subtyping *Pneumocystis* isolates is single-strand conformation polymorphism (SSCP) analysis. This method is based on the concept that specific changes in nucleotide sequence can affect the migration of the fragment when analyzed by gel electrophoresis. To detect these differences, one must denature the double-stranded DNA fragments. The single strands will then fold back on themselves into unique sequence-specific conformations that display different gel migration rates in nondenaturing gels. The resulting fragment patterns can be used for subtyping purposes. Several different SSCP assays have been developed for use with *P. jiroveci*. The first is the rapid SSCP multilocus typing method developed by Hauser and colleagues [47,97,98,113]. In this method, four independent gene loci are evaluated, ITS1, the intron of the nuclear 26S rRNA locus, a variable region of the mtLSU rRNA gene, and a variable region around intron 6 of the beta tubulin gene [47]. This method has been shown to be simple, reproducible, and highly sensitive for detecting strain differences [47,92,98,113]. It has been used to differentiate and evaluate *P. carinii* and *P. ratti*, the two *Pneumocystis* species found in rats [114]. It has been applied to evaluate several questions of epidemiological importance, including the evaluation of prophylaxis failure [115], characterization of genetic diversity among strains from HIV-positive and HIV-negative patients [47], estimating the prevalence of *P. jiroveci* coinfections in patients with *Pneumocystis* pneumonia,[98], and evaluating the possibility of person-to-person transmission [116].

An SSCP application has also been developed for evaluation of the DHPS locus [117]. This assay allows simple, reproducible, and rapid identification of the four different genotypes seen at amino acid positions 55 and 57, which have been evaluated with respect to sulfa exposure, as discussed previously. In quantitating this assay, the authors report the ability to detect coinfecting genotypes that are present in as little as 10% of the overall target population.

SSCP analysis, in comparison to direct DNA sequencing, is generally simpler, less expensive, more rapid, and possibly more sensitive for the detection of coinfecting strains that are present in very low concentration in the target population. One disadvantage, however, is the fact that this method is looking at fragment migration patterns that are derived from the primary DNA sequence, which must first be characterized by sequencing. Consequently, there is a risk that new genotypes, which have not been adequately characterized, may have similar migration

patterns as other genotypes and therefore may not be recognized as new. Additionally, now that transient subclinical carriage has been reported to occur and may be relatively common [22,25], the high incidence of coinfections reported in some studies may simply reflect residual *Pneumocystis* organisms, which may or may not be viable. For this reason, both SSCP and DNA sequencing will probably continue to be used in different laboratories and for different specific applications.

Restriction Fragment Length Polymorphism

One last DNA sequence-derived approach, which utilizes fragment analysis rather than complete sequence determination, is termed restriction fragment length polymorphism (RFLP). This method involves PCR amplification of a designated DNA gene fragment followed by subsequent digestion of the fragment with a restriction endonuclease, which is specific for a unique sequence of nucleotides that may be present or absent within the fragment, depending on polymorphism that exists among different subtypes. This method has been utilized for the evaluation of mutations at the DHPS locus [118] and for subsequent correlation with resistance to sulfa drugs [119]. As with TSO, this method allows rapid, simple, and inexpensive determination of specific genotypes being targeted for evaluation. Consequently, when these are the only genotypes present that are of importance to the investigator, this method is highly useful. No additional information is available, however, about other sequence differences that may be present in other parts of the DNA fragment. Thus this approach is limited in its range of applications.

III. Matching the Gene Locus and Method with the Typing Objective

Depending on the specific goal of the study, certain gene loci and methodological approaches will be better suited for some applications than for others. Some genes, such as the ITS locus, evolve very rapidly and consequently demonstrate much higher rates of polymorphism. This characteristic makes these genes more desirable for evaluating certain questions but less desirable for others. Additionally, some genes are under environmental selection, such as the DHPS locus, while others may be considered neutral in this regard. Whether or not a gene is under selection can have a great impact on the observed polymorphism and subsequent inferences that are drawn. Since multilocus typing is often performed, an additional consideration may include genetic linkage, which is implied for all genes if the organism is a clonal. Since *Pneumocystis* is probably not clonal but sexual, genetic linkage, if it occurs, would be associated with other factors, such as chromosomal proximity of loci. Finally, some genes have sequence-related characteristics, such as high A + T or G + C content or sequence repeat motifs that may result in PCR artifact or other technical difficulties, which must be taken

into account in evaluating the resulting data. This feature is particularly true for ITS1, which contains a 10-bp thymine repeat that routinely introduces PCR artifact in the resulting amplified gene fragment.

Summaries of some of the most commonly examined genes and their corresponding applications are shown in Tables 1 and 2. In general, genes that evolve very slowly are best utilized in studies aimed at evaluating genetic diversity among the different *Pneumocystis* species or for evolutionary studies. Within this context, relatively slowly evolving gene loci include the PCR-amplified fragments of the nuclear 5.8S rRNA, Mt SSU rRNA, SODA, and TS. Genes that evolve more rapidly, however, are necessary for evaluating the possibility of point-source exposure, cluster cases, and direct person-to-person transmission. Because of its rapid rate of evolution, the ITS locus is particularly suited for evaluating these types of questions. Genes that evolve at a more moderate rate are useful for studies focusing on strain variation, including the evaluation of many clinical and epidemiologically important questions. Gene fragments that fall into this category include the PCR-generated regions of the Mt LSU rRNA, EPSP, MSG, and intronic regions of the beta tubulin and 26S rRNA genes. The DHPS and DHFR loci also fall into this category; however, it is important to remember that the DHPS locus is under intense drug-induced selection pressure; consequently, while it is a very useful marker for drug studies, it is probably not a suitable marker for other applications, such as general evolutionary research and certain types of transmission studies.

Other considerations must be given to the selection of the specific methodology employed for subtyping. The most common considerations include cost, technical difficulty, and research objective. If the goal is high throughput evaluation for known mutations, a fragment analysis method, such as SSCP or RFLP is highly suitable. If the desire is to gain sequence information on the entire gene fragment, which then allows the investigator to reexamine the sequence for other nucleotide differences that might not previously have been recognized as important, then DNA sequencing would be the preferred methodology. Rather than suggest one "best method" or "best gene" for all epidemiological investigations with *Pneumocystis*, the current situation today, where different approaches are being utilized in different laboratories, is probably more useful and informative to the research and clinical communities.

IV. Contributions of Molecular Typing to Epidemiological and Clinical Issues in *Pneumocystis* Pneumonia

Over the last 15 years through the use of molecular tools and approaches, a great amount of information has been gained about the genetics of the various *Pneumocystis* spp., which has very significantly affected our understanding of

the biology of the organisms and epidemiology of *Pneumocystis* pneumonia in humans. This topic has been the subject of several recent review articles [5–7,120–122]. There are four key areas where these studies have had a particularly significant impact on the way we now view disease in humans.

A. Taxonomy and Disease Reservoirs

One of the most significant differences is that we now know that *Pneumocystis* is a fungus rather than a protozoan. A fungal identity was originally suggested in the late 1980s, following analysis of ribosomal DNA [123,124] and reconfirmed in subsequent years in every gene sequence observed since that time. Additionally, we know that different mammal species have their own corresponding *Pneumocystis* species. In previous years, these have been referred to as *special forms* or *formae speciales*; now, however, the recommendation has been accepted to recognize each of these special forms as independent biological species [5]. The importance of taxonomy to epidemiology cannot be overstated, owing to the critical relationship between taxonomy, host-specificity, and transmission patterns and risk factors for disease in humans. For example, knowing that *P. carinii* is a species associated only with rats, we know that exposure to rats is not a risk factor for disease in humans.

B. Immediate Source of Infection—Latency vs. Active Acquisition

Prior to the era of AIDS, *Pneumocystis* pneumonia was known primarily as a disease of children. Even today, in many clinical infectious disease textbooks, the suggestion is made that *Pneumocystis* is contracted during the early years of life and remains latent, only to be activated in persons with AIDS whose CD4 + lymphocyte counts drop below, 200/mm³. We now know that this is probably not the case at all, but that active transmission of *P. jiroveci* occurs. There are three independent lines of evidence in support of this view, all resulting from molecular studies. The first line of evidence was the observation that recurrent episodes of *Pneumocystis* pneumonia were associated with different *P. jiroveci* strains [12,13,42,43]. These studies reported that in a high percentage of recurring of *Pneumocystis* pneumonia, episodes, the patients had *P. jiroveci* genotypes that differed from those seen in previous episodes. Following a thorough consideration of potentially confounding factors, such as detection sensitivity limits and the possibility of lung sampling errors, it was concluded that the different *P. jiroveci* genotypes were the result of de novo infections that were actively acquired rather than clinical relapses. Consequently, at least in the case of secondary *Pneumocystis* pneumonia, active transmission is of great importance. A second line of evidence comes from a study examining geographic variation in *P. jiroveci* genotypes from patients in different U.S. cities [16]. In this study, it was observed

that patterns in geographic variation were associated with the patients' place of diagnosis and not with their place of birth, suggesting that these infections represented recently acquired *Pneumocystis* pneumonia rather than activated latent infections. A third line of evidence comes from a study on the risk factors for having mutant DHPS genotypes. In this study DHPS mutant genotypes were associated with prior sulfa exposure and with living in San Francisco. Position 55/57 DHPS mutants were observed in over 80% of all cases of *Pneumocystis* pneumonia in San Francisco [61]. Very interestingly, over 53% of AIDS-defining PCP cases of *Pneumocystis* pneumonia presented with mutant DHPS genotypes. Since these patients had not been receiving prophylactic sulfa, the mutant genotypes could not have resulted from sulfa selection but rather as a result of directly acquiring a mutant *P. jiroveci* strain from another individual. Taken collectively, these molecular data strongly suggest that *P. jiroveci* is transmitted directly from person to person and that active acquisition, rather than latency and activation, accounts for the vast majority if not all of these infections seen in adults.

C. The Natural History of *Pneumocystis* pneumonia in Humans

Given the likelihood that *P. jiroveci* is transmitted from person to person within the context of the AIDS epidemic, as a disease entity that has coemerged with AIDS, the question then arises regarding the natural history of *Pneumocystis* pneumonia in humans. As stated earlier, prior to the AIDS epidemic, *Pneumocystis* pneumonia was known primarily as a disease affecting malnourished and premature infants. More recent studies further support the idea of *Pneumocystis* pneumonia as a pediatric disease [2,21,125–127].

An interesting hypothesis for the general epidemiology and natural history of *Pneumocystis* pneumonia can be posited based on studies of *Pneumocystis* in other mammal species and corroborated in humans by recent DNA-based work [2]. Infants are probably the natural host for the disease, becoming colonized in the first few months of life as maternal antibody wanes. This appears to be the case with rabbits, where the young serve as the primary dispersal host for the agent [128–131]. Similar observations in rats have also been reported [132]. In humans, it also appears to be the case, based on studies that report finding either the organism itself or *Pneumocystis* DNA by PCR amplification to be present in clinical or autopsy specimens [21,133,134]. In the majority of immunocompetent healthy individuals, the infection is apparently mild if not subclinical; however, the possibility of clinical disease should be evaluated further [21,135]. As the infant begins to mount its own protective immunity, the infection is probably cleared. Throughout life, *P. jiroveci* exposures likely occur, resulting in transient colonization. The source of exposure for infants as well as adults is probably other individuals, either with active *Pneumocystis* pneumonia or with transient

subclinical infection [17,22,45,48,110,136]. With the advent of AIDS, a second transmission cycle appears to have emerged, primarily involving HIV-infected individuals. Evidence supporting this suggestion is provided in the genotype differences observed in *P. jiroveci* sequences examined in adult HIV-positive patients compared with those seen in immunocompetent infants [134]. In HIV-infected individuals, colonization probably occurs in the same way as in other persons, with progression to clinical disease occurring as CD4+ lymphocyte counts drop with the increasing progression of AIDS. Individuals are treated and recover, only to become recolonized later [12,13,42,43]. Progression to disease, in this circumstance, is apparently governed primarily by the use of antiretroviral therapy (ART) and/or by secondary *Pneumocystis* pneumonia prophylaxis. As CD4+ lymphocyte populations rise subsequent to the use of ART and transient colonization occurs as it does in other persons, the infection either persists or is self-limiting, depending on the immune status of the individual. This is only a hypothesis; however, it is consistent with data observed to date.

D. Molecular Typing and Clinical Correlates

The final area to be discussed is the use of molecular approaches for addressing various clinical questions. Typically, these studies have sought to identify specific markers or mutations that correlate with clinically important outcomes, such as disease severity, prophylaxis failure, or resistance to a given treatment regimen. Two studies have used subtyping of “housekeeping” genes in an effort to identify *P. jiroveci* strains associated either with increased virulence or *Pneumocystis* prophylaxis failure. One study showed a link between the nuclear rRNA ITS genotype A₂C₁ and disease severity, as measured by arterial oxygen tension and the need for adjunct steroid therapy [31]. The same study showed that ITS genotype B₂a₁ was associated with persistent infections. This study suggested that in addition to host-related factors, such as CD4 lymphocyte count and viral load, strain-specific factors could be also involved in the severity of disease. Another study utilized SSCP multilocus typing to evaluate various clinical correlates [115]. These investigators reported an association between SSCP multilocus genotype 6 and the failure of prophylaxis. This genotype was genetically related to ITS B₂a₁ of the previously mentioned study, suggesting a potential linkage between strains containing the specific ITS genotype and persistence and possible loss of sensitivity to anti-*Pneumocystis* drugs. Further studies are needed to verify this link and to characterize the specific mechanisms that may be involved.

The remaining studies have looked at specific gene targets of anti-*Pneumocystis* drugs in an effort to demonstrate a direct correlation between specific mutations and loss of sensitivity to the specific antimicrobial agent. As mentioned earlier, the gene loci evaluated include DHPS (targeted by sulfonamides and sulfones), DHFR (targeted by trimethoprim, pyrimethamine, and related ana-

logues), and Mt CytB (targeted by atovaquone). The greatest amount of effort by far has focused on DHPS because of the strong link between nonsilent mutations at the gene level and sulfa exposure [53,61,62,64,65], prophylaxis failure [57,137], treatment failure [59,63], and death [60] in patients with *Pneumocystis* pneumonia. Since this topic has been discussed in an earlier section of this chapter that focused on individual genes, the actual data are not repeated here. It is very important, however, to recognize that the global observations of these mutations, which appear to be occurring in an increasing frequency, undoubtedly represent the first stages of emergence of drug resistance in *P. jiroveci* and should therefore be monitored very carefully.

V. Conclusion

Molecular biology has had a tremendous impact on our understanding of the biology of the different *Pneumocystis* species and the epidemiology of PcP in humans. Advances in this area have allowed us to determine that *Pneumocystis* is not a single protozoan species but rather a diverse fungal genus that contains numerous species each of which are presumably highly adapted to different mammalian hosts. Furthermore, we know now that *P. jiroveci* is highly specific for humans and does not cross-infect other mammals. Through molecular epidemiological investigations, we have learned that humans are the reservoirs for *P. jiroveci* and presumably become transiently colonized throughout their lives but that long-term latency cannot account for the vast majority of infections seen in HIV-positive individuals. Strong evidence exists in support of persons-to-person transmission, presumably through aerosol. We also see evidence that resistance to sulfa-containing drugs appears to be in the process of emerging.

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Current Regimens For Treatment and Prophylaxis of *Pneumocystis jiroveci* Pneumonia

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I. Introduction

Pneumocystis jiroveci pneumonia was an uncommon disease, occurring in immunocompromised patient populations, until the beginning of the 1980s, when it emerged as a significant cause of morbidity and mortality among HIV-infected patients [1,2]. The major chemotherapeutic agents available for treatment of *P. jiroveci* pneumonia before the AIDS epidemic were parenteral pentamidine and trimethoprim-sulfamethoxazole (TMP-SMX) [3]. The worldwide AIDS epidemic of the past two decades has intensified efforts to develop new chemotherapeutic agents and new regimens for treatment and prophylaxis. To date, however, TMP-SMX, due to its well-established safety and efficacy, as well as its low cost, remains the drug of choice for both treatment and prophylaxis of *P. jiroveci* pneumonia. The new drugs provide alternatives for patients who are intolerant of TMP-SMX or who fail this regimen.

Management of *Pneumocystis* pneumonia (PcP) has improved dramatically over the past 30 years. Periods of immunological susceptibility have been defined for many *Pneumocystis*-susceptible populations, and chemoprophylaxis is used routinely during these periods. If an episode of *Pneumocystis* pneumonia does occur, the prognosis has improved substantially due to earlier diagnosis and earlier

institution of therapy [4–7]. Furthermore, diagnosis has been facilitated by less invasive techniques, such as bronchoalveolar lavage and induced sputum sampling, as opposed to open lung biopsies, and newer, more sensitive methods to identify the organisms have been developed [8–11]. The use of adjunctive corticosteroid treatment for moderate to severe *P. jiroveci* pneumonia has also led to a decrease in mortality [12].

Considerable attention has been given to defining periods of immunological susceptibility to *Pneumocystis* pneumonia [13–29]. Guidelines for prevention (based on the period of susceptibility) and therapy have been developed for certain populations, especially HIV-infected patients [24,27,30]. As a consequence, the number of cases of AIDS-related *Pneumocystis* pneumonia has declined in western Europe and North America [31–34], and chemoprophylaxis is utilized in other susceptible populations. However, *Pneumocystis* pneumonia continues to cause morbidity and mortality for a number of reasons [35]. Some patients with HIV infection are not aware that they are infected or are not receiving medical care. Additionally, some patients with HIV infection or other immunological disorders are not given chemoprophylaxis, do not adhere to their regimens, or develop *Pneumocystis* pneumonia despite chemoprophylaxis.

II. Treatment

There are extensive data on the efficacy of several different regimens for the therapy of acute *Pneumocystis* pneumonia, as well as extensive data concerning the use of adjunctive corticosteroids for AIDS associated *Pneumocystis* pneumonia. Unfortunately, despite almost a half century of experience, there is little concrete information to guide strategies for patients who do not respond to their initial regimen. The recognition that sulfonamide resistance appears to be emerging is likely to influence therapeutic strategies in the future [35,36].

Table 1 summarizes the various treatment regimens for *P. jiroveci* pneumonia.

A. Trimethoprim-Sulfamethoxazole

TMP-SMX is the agent of choice for treatment of acute *Pneumocystis* pneumonia in patients without a history of life-threatening adverse reactions [37]. TMP-SMX is a combination of two antimicrobial agents acting at separate enzymatic sites to inhibit folate metabolism. *Pneumocystis*, like many other microorganisms, is capable of de novo synthesis of folates but lacks the ability to scavenge folates from the environment. In contrast, mammalian cells can utilize preformed folates but not synthesize them de novo. SMX inhibits de novo synthesis of folates by inhibiting the enzyme dihydropteroate synthase (DHPS), which utilizes para-aminobenzoic acid (PABA) to synthesize dihydropteroate, an intermediate metab-

Table 1 Therapeutic Regimens for *Pneumocystis jiroveci* Pneumonia

Drug	Route	Dose	Comments
First choice			
Trimethoprim-sulfamethoxazole	PO	320/1600 mg q8h (2 double-strength tablets q8h)	For patients other than normal-weight adults:
	IV	TMP 5 mg/kg plus SMX 25 mg/kg q8h	Adjust dosage to equal 5 mg/kg TMP q8h. Adjunctive leucovorin is not recommended.
Alternatives			
Pentamidine	IV	4 mg/kg qd max. 300 mg qd	Infused slowly over 1–2 hr in 5% glucose. High rate of toxicity.
Dapsone plus trimethoprim	PO	100 mg qd	
	PO	320 mg q8h	
Atovaquone	PO	750 mg bid	For mild disease. Well tolerated.
Clindamycin plus primaquine	IV, PO	300 mg q6h to 600 mg q8h	May induce hemolysis in G6PD deficiency.
	PO	15 to 30 mg qd	
Trimetrexate plus leucovorin	IV	45 mg/m ² qd	Can be used when IV therapy is indicated and TMP-SMX and pentamidine cannot be used.
	IV, PO	20 mg/m ² q6h	
Adjunctive therapy			
Prednisone	IV, PO	40 mg q12h for 5 days 40 mg qd for 5 days 20 mg qd for 11 days	Documented to be effective if room air PaO ₂ < 70 mmHg within 72 hr of initiating therapy; often used if room air PaO ₂ < 70 mmHg at any point during the illness.

olite. Since mammalian cells lack the DHPS enzyme, SMX has long been an ideal antimicrobial agent. TMP inhibits the reduction of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase. The main effects of these agents, functionally, is inhibition of thymidine synthesis, and thereby DNA synthesis. While mammalian cells also rely on this pathway, DHFR is species-specific, and the affinity of TMP for the human DHFR enzyme is relatively poor, allowing

the use of this compound as an antimicrobial agent. Two agents targeting the same metabolic pathway at different sites have a theoretical potential to act synergistically and to reduce the likelihood of development of resistance. However, animal studies suggest that the anti-*Pneumocystis* effect of this drug combination is primarily based on the sulfa component [38].

The efficacy of TMP-SMX for treatment of *P. jiroveci* pneumonia was first reported by Hughes et al. in 1974. In a subsequent prospective randomized study reported by the same group comparing TMP-SMX to the former drug of choice, intravenous pentamidine, TMP-SMX had equivalent efficacy but fewer adverse reactions, as well as the advantage of oral or intravenous administration [3,39].

Numerous subsequent studies have confirmed the safety and efficacy of TMP-SMX for treatment of *P. jiroveci* pneumonia in HIV-infected and HIV-uninfected patients. Response rates for TMP-SMX have ranged from 60 to 90% [40–52]. The variability in reported efficacy has been due to differences in patient selection, severity of disease, and study design (e.g., crossover to other regimens).

In the 1980s, several studies compared TMP-SMX with parenteral pentamidine, specifically in patients with HIV infection [40–43]. In the only noncrossover trial, Sattler et al. reported a better survival rate in the TMP-SMX group [42]. However, when all the trials are considered together, the two drugs appeared to be equivalent in terms of efficacy. Because of the less serious adverse events related to TMP-SMX and the easier route of administration, TMP-SMX is considered the drug of choice. No agent has to date been shown to have a higher efficacy than TMP-SMX.

The high rate of adverse reactions to TMP-SMX is the major disadvantage of the drug and was the main reason for the need to develop alternative drug regimens before the issue of sulfa resistance arose. The rate of toxicity of TMP-SMX is higher in HIV-infected patients than in uninfected HIV patients [5], though the reasons for this are unclear. HIV infection itself appears to render patients more susceptible to developing idiosyncratic reactions to TMP-SMX and other drugs [53]. Adverse reactions occur in more than 80% of patients and may be dose-limiting in more than 50%. They generally occur after the first week of therapy. The most common toxicities include gastrointestinal symptoms (nausea, vomiting, anorexia), skin rash, fever, cytopenia, hepatitis, nephritis, hyperkalemia, and pancreatitis [54–57]. Skin reactions can range from mild to life-threatening (e.g., Stevens-Johnson syndrome, toxic epidermal necrolysis). Hyperkalemia is caused by the trimethoprim component and is related to inhibition of the potassium efflux pump [58,59].

Almost all the adverse reactions are rapidly reversible following the discontinuation of TMP-SMX, and many (e.g., mild rash, fever) will resolve even if treatment is continued. Leucovorin has been coadministered with TMP-SMX in an attempt to minimize the potential bone marrow-suppressive effects of trimethoprim on the host. One prospective, randomized (but underpowered) trial found an

increased therapeutic failure rate, as well as higher mortality, in patients receiving leucovorin. Based on these suggestive but not definitive data, use of leucovorin in conjunction with TMP-SMX is not recommended [60,61]. Furthermore, it is not clear that leucovorin decreases the incidence of myelosuppression [60–62]. There are some indications that adjunctive corticosteroids administered for treatment of hypoxemia may reduce the frequency of adverse effects related to TMP-SMX [63]. Some investigators recommend monitoring serum drug concentrations of TMP-SMX and adjusting the dosage to achieve serum levels of 5 to 8 $\mu\text{g/mL}$ TMP and 100 to 150 $\mu\text{g/mL}$ SMX to minimize toxicity while maintaining efficacy [42,64]. One study that monitored TMP levels and adjusted dosing to maintain trough levels between 5 and 8 $\mu\text{g/mL}$ found that all patients were able to complete a course of therapy with TMP-SMX with no apparent loss of efficacy. Data supporting such monitoring in terms of improving efficacy or decreasing toxicity are thus sparse. While this approach is logical, it cannot be recommended as a standard of care. There is no standardized, widely available in vitro culture system for human pneumocystis; thus, MIC values of drugs that are active against *P. jiroveci* cannot be determined [65].

Should patients who have experienced prior adverse effects to TMP-SMX be rechallenged with therapy? Most investigators have found that patients with prior nonlife-threatening and non-IgE mediated adverse reactions can be safely rechallenged [56,66]. Leoung et al. reported a prophylaxis study of patients with prior nonsevere adverse reactions who were rechallenged with TMP-SMX, either with a full dose regimen or through dose escalation. The study found that 75% of the dose-escalation group were able to continue to receive TMP-SMX prophylaxis daily for 6 months, compared to only 57% of the direct-rechallenge group [67]. A study by Para et al. supported these findings; thus, it is safe to rechallenge patients with prior nonsevere adverse reactions to TMP-SMX [68]. Dose-escalation regimens appear to be useful, although this is more relevant to prophylaxis than to acute therapy [68–70]. In acutely ill patients, therapeutic levels will usually have to be achieved quickly.

Resistance of *P. jiroveci* to sulfamethoxazole has been suggested, based on molecular approaches [35,36]. Mutations have been detected in the gene encoding the enzyme DHPS, the target of sulfa and sulfone drugs. Mutations can occur at six nucleotide positions and are nonsynonymous, resulting in amino acid changes [71]. Two of these mutations, encoding amino acids at positions 55 and 57, are located in the active site of the enzyme. Mutations at homologous positions have been shown to cause sulfa resistance in *Mycobacterium leprae* [35,72]. Several subsequent studies have confirmed the occurrence of these two point mutations in *P. jiroveci*, either alone or simultaneously, and most studies found a correlation of DHPS mutations with prior sulfamethoxazole or dapsone exposure, primarily during prophylaxis. This suggests evolutionary selective pressure of sulfa drugs and, hence, a pharmacologically significant effect [73–77]. Interest-

ingly, Ma et al. found no mutations in the gene encoding the trimethoprim drug target, DHFR, in a study where DHPS mutations were detected among the *P. jiroveci* isolates [76]. This is consistent with the concept from animal studies that trimethoprim is inactive against *Pneumocystis* [38].

Are DHPS mutations clinically relevant? Helweg et al. found that patients with mutant *Pneumocystis* DHPS and *Pneumocystis* pneumonia were significantly less likely to survive 3 months than patients with wild-type DHPS, though the cause of death was not necessarily related to *Pneumocystis* pneumonia [74]. A number of investigators have also found that patients failing prophylaxis or treatment with sulfa-containing regimens were more likely to be infected with mutant strains [73,75,78]. Not all investigators, however, have been able to find that wild-type DHPS is associated with better outcome [77,79]. Currently, there is not enough evidence to recommend that the presence of *P. jiroveci* DHPS mutations should be used as a guideline for choice of anti-*Pneumocystis* prophylaxis or therapy. Higher-level resistance may emerge in the future, however. Chapter 24 offers a detailed discussion of resistance.

The standard adult dosage of TMP-SMX for *P. jiroveci* pneumonia is 15 to 20 mg/kg per day of trimethoprim plus 75 to 100 mg/kg per day of sulfamethoxazole divided in three to four doses administered orally or intravenously. An oral regimen of trimethoprim 320 mg plus sulfamethoxazole 1600 mg (equaling two double-strength 160/800 mg tablets) three times daily is often used for average-size adults. The oral route can be used to treat mild to moderate (i.e., initial $\text{PaO}_2 > 70$ mmHg breathing room air) cases of *P. jiroveci* pneumonia when no obvious gastrointestinal dysfunction is apparent and patients are likely to be adherent [80,81]. All patients with severe disease (i.e., $\text{PaO}_2 < 70$ mmHg) should be placed on an intravenous regimen and should receive adjunctive corticosteroid therapy (see below). Recommended duration of antimicrobial therapy is 3 weeks for HIV-infected patients, and 2 weeks for HIV-uninfected patients. However, these recommendations are not based on robust data but, rather, on clinical experience, especially from early in the AIDS epidemic.

B. Dapsone plus Trimethoprim

Dapsone is a sulfone drug that inhibits DHPS, and thereby folate metabolism, much like sulfamethoxazole. Dapsone has been used since the mid-1980s in combination with trimethoprim to treat *P. jiroveci* pneumonia [82]. In a double-blind randomized study, dapsone-TMP was compared to TMP-SMX in 60 HIV-infected patients with mild to moderate *P. jiroveci* pneumonia with similar efficacy, but with a lower rate of major adverse effects (30 vs. 57%) [49]. A subsequent double-blind, randomized study in 181 patients with mild to moderate *P. jiroveci* pneumonia comparing oral TMP-SMX, dapsone-TMP, and clindamycin-primaquine did not demonstrate any differences among the regimens with

regard to efficacy or dose-limiting toxic effects [51]. However, the power of each study was not sufficient to exclude clinically significant differences. Overall, dapsone-TMP has similar efficacy as TMP-SMX in mild to moderate *P. jiroveci* pneumonia, and the former may be associated with less frequent adverse reactions than the latter, but dapsone-TMP is currently considered a second-line drug. The regimen for therapy of *P. jiroveci* pneumonia is trimethoprim 320 mg three times daily plus dapsone 100 mg daily orally. This regimen is not as convenient as TMP-SMX because the drugs are not available in a fixed-combination tablet.

Adverse reactions to dapsone-TMP include rash, fever, cytopenia, hyperkalemia, and gastrointestinal symptoms. Many patients who cannot tolerate sulfonamides will tolerate dapsone [69,83]. However, patients with a history of immediate hypersensitivity to one should not receive the other. Asymptomatic methemoglobinemia is commonly associated with dapsone, but clinically important methemoglobinemia appears to be rare [49]. Dapsone can cause hemolysis in patients with glucose-6-phosphate-dehydrogenase (G6PD) deficiency, but routine screening for G6PD deficiency before instituting therapy is probably not warranted unless patient history or ethnicity indicates a potential risk [80].

Dapsone monotherapy (100 mg daily for 3 weeks) has also been investigated. The efficacy of this regimen appears to be lower than the combination of trimethoprim and dapsone; thus, dapsone alone cannot be recommended [84,85].

C. Trimetrexate plus Leucovorin

Another chemotherapeutic compound targeting folate metabolism is trimetrexate. Trimetrexate is a derivative of methotrexate. Both are potent inhibitors of the *P. jiroveci* DHFR enzyme, but methotrexate is not taken up by the organism and is thus inactive. Trimetrexate is effective for treating *P. jiroveci* pneumonia but is available only in an intravenous formulation [86,87]. Because trimetrexate also inhibits human folate metabolism, leucovorin must be administered concomitantly to prevent cytopenias.

Trimetrexate has been compared to TMP-SMX in a double-blind study in 215 AIDS patients with moderate to severe *P. jiroveci* pneumonia [87]. The study showed that although trimetrexate is effective against *P. jiroveci* pneumonia, it is less effective, but better tolerated, than TMP-SMX. The dosage recommended for treatment of *P. jiroveci* pneumonia is trimetrexate, 45 mg/m² intravenously once daily, plus leucovorin 20 mg/m² orally or intravenously four times daily. Trimetrexate can be used when intravenous treatment is warranted but the patient cannot tolerate or fails intravenous TMP-SMX and pentamidine [80]. There is no indication that the coadministration of leucovorin impairs the efficacy of trimetrexate against *P. jiroveci* [81], despite the suggestion that leucovorin impairs the efficacy of TMP-SMX [60,61].

Adverse effects due to trimetrexate are uncommon except for myelosuppression.

D. Pentamidine

Pentamidine belongs to the diamidine class of compounds and has antiprotozoan activity, in addition to activity against *Pneumocystis*. The mode of action probably involves binding to DNA, although details about the drug's activity against *P. jiroveci* are unknown. It was the first drug reported to treat *P. jiroveci* pneumonia successfully. Subsequent reports have confirmed the efficacy of intravenous pentamidine; it appears to be equivalent in efficacy to TMP-SMX for treatment of *P. jiroveci* pneumonia [3,39–43,46,88,89].

The recommended dose of intravenous pentamidine is 4 mg/kg once daily (maximum 300 mg daily), infused slowly over 1 to 2 hr in 5% glucose. Intramuscular administration was used for many years, but this was found to produce impressive sterile abscesses at the injection site. Intravenous administration had been thought to be unsafe, but subsequent studies showed that slow administration was quite safe. Prolongation of the QT interval and subsequent risk of ventricular arrhythmias, in particular torsades de pointes, has been reported and electrocardiographic (ECG) monitoring suggested [90]. A subsequent study did not find an association between pentamidine therapy and an increase in the QT interval or the occurrence of ventricular arrhythmias [91]. Both studies were prospective but included a limited number of patients receiving pentamidine (Refs. 14 and 16, respectively); the differences in the observations may relate to the duration of therapy, severity of disease, or changes in the management of *Pneumocystis* pneumonia (e.g., use of adjunctive corticosteroids). While some experts recommend that intravenous pentamidine be administered with ECG monitoring, the risk of arrhythmias appears to be sufficiently low; therefore, most experts do not recommend this.

Pentamidine is a relatively toxic drug; 80% or more of patients experience some sort of adverse reaction. Discontinuation due to toxicity occurs in almost half of the cases [3,41–43,55]. Toxic effects include nephrotoxicity, dysglycemia, pancreatitis, cytopenia, and cardiac adverse effects (long QT and arrhythmias). Hypoglycemia can occur at any time during therapy or many weeks after its discontinuance. Patients may also become hyperglycemic due to pentamidine-induced toxicity to islet cells of the pancreas. Accumulation of the drug occurs during therapy due to the drug's long half-life. This poses a potential problem when discontinuation is warranted due to toxicity.

Aerosolized pentamidine has been assessed as therapy for acute AIDS-associated *Pneumocystis* pneumonia. Aerosolized pentamidine is well tolerated but not nearly as effective as intravenous pentamidine or TMP-SMX [92,93]; thus, it is not recommended for use as therapy of *Pneumocystis* pneumonia.

Pentamidine is the main initial alternative when intravenous therapy is warranted and the patient has failed therapy with TMP-SMX or is intolerant of it.

E. Atovaquone

Atovaquone targets the cytochrome B complex and, thus, inhibits mitochondrial electron transport [50,94,95]. This drug was developed clinically in the 1990s. It is available only as an oral agent. Atovaquone is a second-line agent for mild *P. jiroveci* pneumonia if TMP-SMX cannot be used.

In a large double-blind trial, atovaquone was compared to oral TMP-SMX in mild to moderate AIDS-related *P. jiroveci* pneumonia [50]. Mortality after 4 weeks was higher in the atovaquone group (11 deaths vs. 1 death); similarly, other parameters of response to therapy favored TMP-SMX. However, atovaquone was better tolerated, with fewer treatment-limiting adverse events, and is similarly better tolerated than intravenous pentamidine [50,95]. Most studies have investigated a tablet formulation of atovaquone, but an oral suspension that is more reliably absorbed is now available. Since the response rate is related to the atovaquone level, the suspension formulation should provide improved efficacy [96].

The most common adverse effects of atovaquone are rash and gastrointestinal symptoms.

Resistance to atovaquone may be emerging [97]. In one study, mutations at the coenzyme-Q binding site of cytochrome B was detected in 2 of 4 *P. jiroveci* isolates from patients who had prophylaxis failure on atovaquone, and in 2 of 10 patient isolates overall. Further studies are needed, however, before any conclusions can be drawn. Chapter 24 offers a detailed discussion on this topic.

The standard dosing regimen is atovaquone 750 mg orally twice daily. Of note, the drug should be taken with fatty foods to increase gastrointestinal absorption.

F. Clindamycin plus Primaquine

Clindamycin-primaquine has been evaluated as primary and salvage therapy for *P. jiroveci* pneumonia [51,98–100]. A large, but underpowered three-armed study compared clindamycin-primaquine to TMP-SMX and dapsone-TMP. No differences in efficacy or toxicity were identified [51]. Another small double-blind trial comparing clindamycin-primaquine to TMP-SMX found the drugs to have similar efficacy, but the clindamycin-primaquine regimen was found to be associated with fewer adverse effects [100].

A recent metaanalysis suggests that clindamycin-primaquine may have higher efficacy than any other salvage regimen in patients failing primary therapy [101]. However, this retrospective analysis is based on limited data and must be interpreted with caution, since selection bias may have led to the use of this agent in less severely ill patients.

The standard dosage of this regimen is clindamycin 300 to 600 mg orally or intravenously, three to four times daily, plus primaquine 15 to 30 mg orally once daily.

Most common adverse effects include rash, fever, antibiotic-associated diarrhea (*Clostridium difficile*), and hepatitis. Also, primaquine may induce hemolysis in G6PD-deficient patients.

G. Other Agents

Novel agents undergoing preclinical investigation include echinocandins and pneumocandins, which target synthesis of $\beta = 1,3$ glucan, a cell wall compound of *Pneumocystis* and other fungi [102,103].

H. Adjunctive Corticosteroid Treatment

Patients with *P. jiroveci* pneumonia frequently experience worsening of their pulmonary function in association with institution of anti-*P. jiroveci* treatment, resulting in decreased oxygenation and progression of lung infiltrates. This may be a Jarisch-Herxheimer–like reaction caused by the release of toxic compounds from dead organisms, increasing the inflammation in the pulmonary tissue. A worsening of oxygenation is particularly critical for patients with initial marked hypoxemia and may lead to a rapid deterioration with adult respiratory distress syndrome (ARDS) and death [104,105]. Therefore, adjunctive corticosteroids have been investigated in *P. jiroveci* pneumonia. Studies have documented the beneficial effects of administering corticosteroids to patients with severe disease (i.e., $\text{Pa}_{\text{O}_2} < 70$ mmHg within 72 hr of initiation of antimicrobial therapy); such therapy reduces mortality rates, as well as recovery time [106–109]. Adjunctive corticosteroid treatment is considered the standard of care for patients with *P. jiroveci* pneumonia presenting with a $\text{Pa}_{\text{O}_2} < 70$ mmHg while breathing room air [105]. The recommended regimen is a 21-day course of oral prednisone (40 mg twice daily for 5 days, 40 mg daily for 5 days, and 20 mg daily for 11 days). Patients with a $\text{Pa}_{\text{O}_2} > 70$ mmHg also benefit from adjunctive corticosteroids in terms of pulmonary physiology, but survival outcome has not been shown to improve. Demonstrating such improvement would require a large study, given the low mortality in this group.

Interestingly, one study assessing markers of prognosis of *P. jiroveci* pneumonia in the era of adjunctive corticosteroid treatment did not find Pa_{O_2} to be independently associated with poor outcome, which could possibly be explained in part by the beneficial effects of adjunctive corticosteroid treatment of patients with low Pa_{O_2} [110]. However, another study looking at the corticosteroid era did find that $\text{Pa}_{\text{O}_2} < 60$ mmHg was associated with an increased mortality rate [111].

Despite theoretical concerns, there is no convincing evidence that the 21-day course precipitates other opportunistic infection or exacerbates tuberculosis [12,63,112].

I. Notes on Treatment

The course of treatment most often recommended is at least 21 days for HIV-infected and 14 days for non-HIV-infected patients, regardless of the drug regimen being used. Courses longer than 21 days are sometimes recommended for patients whose disease is severe or slow to respond.

When an HIV-infected patient develops *P. jiroveci* pneumonia or another serious opportunistic infection, the question often arises whether to initiate or continue HAART. It seems logical to assume that the patient would benefit from the potential immune-augmenting effects of HAART in clearing the opportunistic infection (OI). However, a paradoxical response could occur because of tissue damage due to an enhanced inflammatory response [113]; for example, HAART-related immune-reactivation retinitis has been reported [114]. When patients have acute *Pneumocystis* pneumonia, oral absorption of HAART drugs may not be reliable. In addition, adherence may be difficult and drug interactions may be difficult to predict. Furthermore, when a toxicity occurs, it may be difficult to determine whether it is due to an antiretroviral HAART agent or an agent used to treat *Pneumocystis* pneumonia. Therefore, as a result, caution should be used in administering HAART until the episode pneumonia has resolved.

When to switch therapy in failing patients and what drug regimen to switch to as salvage therapy have long been perplexing problems. Generally, cases of *P. jiroveci* pneumonia resolve slowly. Improvement in symptoms, signs, and physiological parameters is usually seen in 4 to 8 days [80]. Therefore, changes in treatment due to lack of efficacy should rarely be made prior to 4 to 8 days. It seems prudent to rule out concurrent infectious or noninfectious processes as the cause of the deterioration. Bronchoalveolar lavage and assessment for congestive heart failure and pulmonary emboli are warranted. Many experts would assess for other causes of pulmonary dysfunction and then, after 4 to 8 days of TMP-SMX, switch to pentamidine. Alternatively, pentamidine may be added to TMP-SMX, although this approach has not been systematically examined. If there is a contraindication to pentamidine, or if the patient fails to improve after 4 to 8 days of pentamidine, trimetrexate is the preferred next option. Clindamycin-primaquine can be used, but there is no parenteral form of primaquine available for severely ill patients.

III. Prophylaxis

A strategy to prevent *Pneumocystis* pneumonia could include prevention of exposure, antimicrobial chemoprophylaxis, and immune modulation [35].

There is much still to be learned about the life cycle and mode of transmission of *Pneumocystis*. Airborne transmission has been documented in animal models, and *Pneumocystis* DNA has been detected in ambient air. Thus, it is likely, that human *P. jiroveci* also is spread by an aerosol route [115–119]. It would seem reasonable to isolate patients with *P. jiroveci* pneumonia from other susceptible patients, even though no patient-to-patient transmission has been unequivocally documented. Recent molecular studies suggest that patient-to-patient transmission may occur, and that healthy subjects may become colonized through close contact with patients who have *Pneumocystis* pneumonia [120–122]. Current guidelines do not recommend isolation of these patients, but such an approach is logical. Many hospitals isolate patients with *Pneumocystis* pneumonia from susceptible individuals in both outpatient and inpatient settings.

For cancer and transplant patients, reducing the degree of immunosuppression and its length in an effort to minimize the risk of opportunistic infections is a logical strategy to pursue. Given the life-threatening nature of the underlying illness, such an approach is often difficult to achieve. The immune reconstitution in HIV-infected patients responding to HAART has a well-documented effect on reducing the risk of opportunistic infections, including *P. jiroveci* pneumonia. To date, there have not been any studies in humans to evaluate passive or active immunization against *P. jiroveci* [35].

A well-conceived strategy for antimicrobial chemoprophylaxis requires defining a population at risk, defining that population's time period of susceptibility, and weighing efficacy against the risk of adverse effects, the effect of chemoprophylaxis on antimicrobial resistance in targeted and nontargeted organisms, and the cost of the prophylaxis. Prophylaxis can be directed either toward the first episode of *P. jiroveci* pneumonia (primary prophylaxis) or recurrent disease (secondary prophylaxis), and should be continued for as long as the immunosuppressive condition persists.

For allogeneic bone marrow transplant patients, current guidelines recommend prophylaxis against *P. jiroveci* pneumonia from time of engraftment until 6 months posttransplant [123].

Hughes et al. reported in the mid-1970s that *P. jiroveci* pneumonia was a frequent occurrence in children with acute lymphoblastic leukemia, with incidence rates of 22 to 45%. In order to reduce its distressing morbidity and mortality, Hughes showed that administration of TMP-SMX successfully prevented *P. jiroveci* pneumonia in this population [25,124]. This work established the importance of prophylaxis in children with malignant neoplasms, especially acute lymphocyte leukemia, and became the basis for using TMP-SMX in other patient populations shown to be at risk by observational data. Unfortunately, except for patients with HIV infection, there are no laboratory parameters that define risk (e.g., CD4 cell counts) [27]. However, prophylaxis is often used in a variety of underlying diseases (Table 2).

Table 2 Risk of *Pneumocystis jiroveci* Pneumonia According To underlying Disease.

Disease	Period	Patients with <i>Pneumocystis</i> Pneumonia (%)
Myeloma	1954–1975	0
Hodgkin's disease	1967–1970	0.04
Acute myelocytic leukemia	1967–1970	0.20
Acute lymphocytic leukemia: 4-drug	1972–1975	22
Acute lymphocytic leukemia: 4-drug + radiation	1972–1975	45
Lymphoma: PROMACE-MOPP	1981–1983	0
Lymphoma: PROMACE-cytaBOM	1981–1983	32
Allogenic bone marrow transplant	1993–1996	4.8
Kidney transplant	1987–1996	0.4
Heart/liver/lung transplant	1987–1996	2–11
HTLV-1 associated lymphoma	1985–1995	50
HIV infection	1980–1990	70–90

Key: PROMACE-MOPP is prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, mustargen, vincristine, and procarbazine; PROMACE-cytaBOM is prednisone, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine, methotrexate, and leucovorin; HTLV-1 is human T-lymphotropic virus 1; HIV is human immunodeficiency virus.

Sources: Adapted from Ref. 35 and based on Refs. 25 and 169 to 174.

Prior to the routine use of prophylaxis during their clinical course, more than 80% of HIV-infected patients were estimated to have at least one episode of pneumonia [125], the risk for which is related to the absolute CD4 cell count [28,29]. A large cohort study found that the risk of contracting *P. jiroveci* pneumonia within 1 year was 18.4% for patients with a baseline CD4 cell count $<200/\text{mm}^3$, versus less than 5% for patients with CD4 cell counts $> 200/\text{mm}^3$ [28]. Persistent fever and oral candidiasis were independent risk factors for *P. jiroveci* pneumonia. A subsequent study has suggested that patients with CD4 cell counts $< 250/\text{mm}^3$, or $<14\%$, should be targeted for prophylaxis [126]. Either approach is probably reasonable.

For patients with HIV infection, current USPHS-IDSA guidelines recommend institution of primary prophylaxis against *P. jiroveci* pneumonia in patients with CD4 cell counts $< 200/\text{mm}^3$ or oropharyngeal candidiasis, and suggest that prophylaxis may be considered in additional patients with a history of prior AIDS-defining illness and CD4 cell counts $< 14\%$ or $<250/\text{mm}^3$ [24]. Some investigators have also considered instituting prophylaxis in HIV-infected patients with a history of substantial unexplained weight loss or unexplained fever > 2 weeks [22]. The viral load of HIV in plasma has been shown to be an independent predictor of the development of opportunistic infections, though data are inade-

quate to predict the occurrence of specific infections in a timely manner; thus, viral load is not included in current guidelines for risk assessment and prevention of *P. jiroveci* pneumonia [22,127].

HIV-infected patients who have recovered from a primary episode of *P. jiroveci* pneumonia are at high risk of developing recurrent disease, unless the CD4 cell count rises to $>200/\text{mm}^3$ due to antiretroviral therapy. Recurrence rates within 6 months without prophylaxis have been found to be approximately 50%; in contrast, 10% of patients receiving chemoprophylaxis relapse [128,129]. Therefore, secondary prophylaxis is the standard of care in the HIV-infected population whose CD4 cell counts remain $<200/\text{mm}^3$. Interestingly, in non-HIV-infected patients, recurrent *Pneumocystis* pneumonia has been reported only infrequently.

Failure of *P. jiroveci* pneumonia prophylaxis is associated with use of an agent other than TMP-SMX for prophylaxis, and with a CD4 cell count $< 50/\text{mm}^3$ [130].

Profound immune reconstitution, with increasing CD4 cell counts and T-lymphocyte function, can be seen in patients responding to HAART with a decrease in viral load [131–135]. This has raised the question of whether the well-established relation between the CD4 cell count and the risk of development of *Pneumocystis* pneumonia is still valid in the HAART era [22]. In particular, investigators have speculated whether low-nadir CD4 cell counts would result in ‘holes’ or ‘blind spots’ to certain opportunistic infections despite the HAART-induced immune reconstitution and associated increase in CD4 cell count [136,137]. A large, multicenter cohort study found that increases in CD4 cell counts to at least $200/\text{mm}^3$ were associated with a substantially reduced risk of opportunistic infections compared to patients whose CD4 cell counts remained below $50/\text{mm}^3$ (3.7 to 8.1 vs. 72.9 episodes per person-year). Nadir CD4 cell counts were associated with only a slightly increased rate of opportunistic infection in the patients whose CD4 cell counts rose above $200/\text{mm}^3$ [13]. This indicates that HIV-infected patients experiencing an increase in CD4 cell counts to above $200/\text{mm}^3$, despite low nadir CD4 cell counts, are protected against opportunistic infections and suggests that prophylaxis may be discontinued in this population.

Several studies have examined the safety of discontinuing primary and secondary *Pneumocystis* prophylaxis in patients responding to HAART [14–21,23]. Based on these studies, it is now evident that it is a safe strategy to discontinue primary, as well as secondary prophylaxis, in patients who have responded to HAART with a sustained (more than 3 months) CD4 cell increase to $>200/\text{mm}^3$ [24]. However, patients receiving secondary prophylaxis who had their prior *P. jiroveci* pneumonia episode occurring at a CD4 cell count $> 200/\text{mm}^3$ should probably receive prophylaxis for life. In patients stopping prophylaxis, it should be reintroduced when CD4 cells count drop below

Table 3 Chemoprophylactic Regimens for Prevention of *Pneumocystis jiroveci* Pneumonia

Drug	Dose	Comments
First choice		
Trimethoprim-sulfamethoxazole	160/800 mg qd (1 DS tablets qd) or 80/400 mg qd (1 SS tablet qd)	1 SS tablet qd as effective as 1 DS tablet qd and associated with fewer side effects. 1 DS tablets tiw is recommended if daily therapy is not tolerated. Rechallenge of patients with prior non-life-threatening adverse effects is recommended; gradual dose escalation may be helpful. TMP-SMX is efficacious in preventing toxoplasmosis.
Alternatives		
Dapsone	100 mg qd	It is unclear whether dapsone alone is protective against toxoplasmosis, while dapsone – pyrimethamine is effective in preventing toxoplasmosis.
Dapsone plus pyrimethamine plus leucovorin	200 mg qwk 75 mg qwk 25 mg qwk	
Dapsone plus pyrimethamine plus leucovorin	50 mg qd 50 mg qwk 25 mg qwk	
Aerosolized pentamidine	300 mg qmo	
Atovaquone	1 500 mg qd	Delivered by Respigard II nebulizer. Not effective in preventing toxoplasmosis. Efficacy in preventing toxoplasmosis is unknown.

Key: DS, double-strength; SS, single-strength; tiw, qwk, per week; qmo, per month.

200/mm³ or if the patient experiences an episode of *P. jiroveci* pneumonia with a CD4 cell count > 200/mm³ [24].

Table 3 summarizes recommended prophylaxis regimens for *Pneumocystis* pneumonia.

A. Trimethoprim-Sulfamethoxazole.

TMP-SMX, one double-strength tablet (160 mg TMP/800 mg SMX) daily, is the drug of choice for preventing *P. jiroveci* pneumonia in HIV-infected, as well as

non-HIV-infected patients [138–140]. In a randomized study comparing TMP-SMX to aerosolized pentamidine in patients with AIDS, none of the patients receiving TMP-SMX developed *P. jiroveci* pneumonia vs. 11% in the aerosolized pentamidine group [141]. Several other studies have confirmed the safety and efficacy of TMP-SMX for prophylaxis against *P. jiroveci* pneumonia [129,142]. Unfortunately, TMP-SMX is associated with a high incidence of adverse reactions over the long term; in one study, 50% of patients switched to another regimen after 3 years [142].

One single-strength tablet of TMP-SMX (80 mg TMP/400 mg SMX) daily is associated with fewer side effects than one double-strength tablet daily, and there is no evidence suggesting that the lower dose is less efficacious in preventing disease [143]. Because of the well-established safety, efficacy, and low cost of TMP-SMX, this regimen should be continued wherever possible. Therefore, administration of daily single-strength TMP-SMX is a reasonable approach to reduce toxicity [140]. Other strategies to reduce treatment-limiting adverse effects include dose escalation when TMP-SMX prophylaxis is being initiated [68]. Likewise, patients who have not experienced life-threatening toxicity may benefit from being rechallenged using dose escalation [67]. Thrice weekly administration of double-strength TMP-SMX does decrease toxicity, but the regimen may be less efficacious [144]. Current guidelines recommend either single- or double-dose TMP-SMX once daily and strongly suggest consideration of rechallenge of patients with prior nonlife-threatening adverse effects [24].

TMP-SMX also confers protection against toxoplasmosis and bacterial respiratory infections [145]. These features make TMP-SMX an attractive option for prophylaxis.

Adverse effects are similar to those seen during therapy with TMP-SMX (see above).

B. Dapsone-containing Regimens

Dapsone or dapsone-pyrimethamine-leucovorin are effective alternative regimens for preventing *P. jiroveci* pneumonia. Dapsone containing regimens are considered by many to be the second choice for patients intolerant of TMP-SMX [80,142,146,147]. However, approximately 20% of patients who are intolerant of TMP-SMX will probably exhibit adverse reactions to dapsone [83]. Dapsone 50 mg twice daily or 100 mg once daily is effective, while lower doses are not recommended [142]. Alternatively, two regimens—[1] dapsone 50 mg daily plus pyrimethamine 50 mg weekly plus leucovorin 25 mg weekly or [2] dapsone 200 mg weekly plus pyrimethamine 75 mg weekly plus leucovorin 25 mg weekly—are also effective. While it is unclear whether dapsone alone is active in preventing toxoplasmosis, dapsone-pyrimethamine is protective [147–149].

Adverse effects are similar to those that occur during therapy (see above).

C. Aerosolized Pentamidine

Aerosolized pentamidine has been evaluated primarily in patients with HIV infection. Aerosolized pentamidine delivered by the Respigard II nebulizer is effective in preventing *P. jiroveci* pneumonia, but is inferior to TMP-SMX [129, 141, 142, 150, 151]. This appears to be especially true in patients with a CD4 cell count $< 100/\text{mm}^3$ [142]. However, it is exceptionally well tolerated; the most common adverse effect is bronchospasm requiring bronchodilators. Aerosolized pentamidine is not active against toxoplasmosis. Patients receiving aerosol pentamidine should be screened for tuberculosis, since outbreaks due to coughing of tuberculosis secretions into the atmosphere have been described [80].

The recommended regimen is 300 mg pentamidine once monthly delivered by the Respigard II nebulizer. Higher doses (i.e., 600 mg monthly or 300 mg twice monthly) have been evaluated only on a limited basis, but may have a role in patients who experience breakthroughs of *P. jiroveci* pneumonia [24, 152].

Intermittent intravenous pentamidine has not been adequately studied as a prophylactic regimen. Because of its uncertain efficacy and the toxicity of cumulative doses, intermittent intravenous pentamidine should be considered only in very unusual circumstances [24].

D. Atovaquone

Atovaquone has been shown in randomized trials to be effective in preventing *Pneumocystis* pneumonia [153, 154]. One trial compared atovaquone to dapsone and found similar efficacy, but atovaquone was better tolerated [153]. In another randomized trial, atovaquone had efficacy similar to that of aerosolized pentamidine, but was associated with more frequent treatment-limiting adverse effects [154]. Thus, atovaquone is a good alternative for patients intolerant of TMP-SMX, but it is considerably more expensive than the other second-line agents. Although atovaquone has been used as salvage therapy in toxoplasmosis, its efficacy in preventing toxoplasmosis has not been assessed.

The recommended regimen is 1500 mg daily (i.e., similar to the total daily dose used for treatment).

Adverse effects are similar to those experienced during treatment.

E. Other Agents

Clindamycin-primaquine has been investigated as prophylaxis for *Pneumocystis* pneumonia, but the results have been disappointing. Thus, this regimen cannot be recommended for prophylaxis [155].

A post hoc analysis of patients receiving prophylaxis against *Mycobacterium avium-intracellulare* complex found that patients receiving azithromycin had fewer episodes of *P. jiroveci* pneumonia than those not receiving azithro-

mycin [156]. All patients in the trial were also receiving specific prophylaxis against *P. jiroveci* pneumonia. The clinical significance of this finding needs to be further investigated, but at this point azithromycin cannot be recommended for prophylaxis against *P. jiroveci* pneumonia.

IV. Treatment and Prophylaxis in Pediatric Patients

Generally, the principles of prophylaxis use and choice of drug regimen for treatment and prophylaxis in the pediatric population are similar to those described above for adults.

In one study, more than half of the cases of *Pneumocystis* pneumonia in perinatally HIV-infected children occurred between 3 months and 6 months of age [157]. Therefore, initiation of prophylaxis is recommended at 4 to 6 weeks of age for all infants born to HIV-infected mothers, to be discontinued if the infant is subsequently found not to be HIV-infected [24]. Prophylaxis is not recommended during the first month of life due to high risk of adverse drug reactions and the small risk of *Pneumocystis* pneumonia occurring within this period. Healthy infants and children have higher CD4 cell counts than healthy adults, and *Pneumocystis* pneumonia can occur in HIV-infected infants and children with CD4 cell counts higher than those in adults [158,159]. Therefore, prophylaxis should be continued for infants of unknown HIV status and HIV-infected infants for the first year of life regardless of CD4 cell count; after that time, age-specific CD4 cell counts are used to assess for continuation of prophylaxis (Table 4) [160]. The safety of discontinuing primary prophylaxis in children receiving HAART is being prospectively addressed in PACTG study P1008. Preliminary data from 236 children followed for approximately 2 years suggest

Table 4 Indications for Chemoprophylaxis in HIV-Exposed or HIV-Infected Infants and Children

Age and HIV Status	PcP Prophylaxis
Birth to 4–6 weeks HIV-infected or unknown	No prophylaxis
4–6 weeks to 1 year HIV-infected or unknown	Prophylaxis
1–5 years	Prophylaxis if CD4 cells < 500/mm ³ or CD4 cell percentage < 15%
6–12 years	Prophylaxis if CD4 cells < 200/mm ³ or CD4 cell percentage < 15%

Source: Adapted from Ref. 160.

that primary prophylaxis can be safely discontinued in children over 6 years of age who have an increase in the CD4 count to greater than 20% for at least 4 months, and in children 2 to 6 years of age who have an increase to greater than 25% for at least 4 months (Dr. Wayne M. Dankner for the PACTG P1008 protocol team, personal communication). Data supporting discontinuation of secondary prophylaxis are unavailable; thus, current recommendations for patients who had an episode of *Pneumocystis pneumonia* in childhood suggest lifelong prophylaxis [24]. Extrapolation from the adult experience would suggest, however, that even secondary prophylaxis can be safely discontinued in children with a sustained immunological and virological response to HAART. As previously addressed, the work by Hughes and colleagues in the 1970s demonstrated the importance of prophylaxis in children with malignant diseases, especially acute lymphocytic leukemia [25].

The recommended drug of choice for prophylaxis is TMP-SMX, with dapsone, aerosolized pentamidine, and atovaquone being alternative drug regimens (Table 5) [24]. TMP-SMX administered three times weekly has been shown to be

Table 5 Chemoprophylactic Regimens for Prevention of *Pneumocystis jiroveci* Pneumonia in Infants and Children

Drug	Dose	Comments
First choice		
Trimethoprim-sulfamethoxazole	75/375 mg/m ² bid 3 days a week on consecutive days	TMP-SMX is efficacious in preventing toxoplasmosis.
Alternatives		
Dapsone	2 mg/kg qd (max 100 mg) or 4 mg/kg qw (max 200 mg)	Daily dosage is more efficacious than weekly dosage in preventing PcP, but has a higher rate of toxicity. It is unclear whether dapsone alone is protective against toxoplasmosis.
Aerosolized pentamidine	300 mg qmo	For children aged ≥ 5 years. Delivered by Respigard II nebulizer. Not effective in preventing toxoplasmosis.
Atovaquone	Aged 1–3 mo. and >24 mo: 30 mg/kg qd Aged 4–24 mo: 45 mg/kg qd	Efficacy in preventing toxoplasmosis is unknown.

Source: Adapted from Ref. 24.

as effective as daily regimens [161]. Daily dapsone is more effective in preventing *Pneumocystis* pneumonia than weekly dapsone, but is associated with a higher rate of toxicity [162]. Aerosolized pentamidine is not recommended for children aged 5 years of age or below due to concerns about their ability to cooperate, but it is effective in preventing *Pneumocystis* pneumonia in children [163–165].

As in the adult, TMP-SMX is also the drug of choice for therapy in the pediatric population. Intravenous pentamidine is an acceptable alternative to patients not tolerating or failing TMP-SMX. One small study comparing TMP-SMX orally to intramuscular pentamidine found similar efficacy [40]. For TMP-SMX, the recommended regimen is intravenous administration of 5/25 mg/kg four times daily. For pentamidine, the recommended regimen is 4 mg/kg intravenously once daily. Experience with alternative drugs is limited, but oral atovaquone, 20 mg/kg twice daily, could be used for patients with mild to moderate *Pneumocystis* Pneumonia not tolerating the other regimens. Dapsone-trimethoprim, clindamycin-primaquine, and trimetrexate-leucovorin have been inadequately studied, but could be utilized based on their activity in adults if other regimens cannot be used. There are indications that adjunctive corticosteroids may be of benefit in the pediatric setting as well, although a limited number of studies have been conducted (166–168).

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21

Lipids

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I. Introduction

Several reports and reviews on *Pneumocystis* lipids have appeared since the last edition of this book [1,2], so there is now ample information available for an entire chapter devoted to this topic. Most biochemical data are on *P. carinii* [3] organisms isolated and purified from infected rat lungs. Less is known about human-derived *P. jirovecii* [4] and other species. All major lipid classes have been examined (Fig. 1); but details on molecular structures and metabolism are available for only some groups, such as the sterols, ubiquinones, and fatty acids.

II. Organism Preparations Used for Lipid Analyses

The absence of culture techniques for supporting luxuriant growth of *Pneumocystis* has been a factor in hindering research on the biochemical and physiological nature of the organism [5]. An axenic culture system has been developed [6] (see also Chap. 22), but cell proliferation is slow and organism yield is less than that obtained from infected lungs. Generally, organisms in culture provide a number of advantages for manipulating experimental conditions, but in vitro cultivation

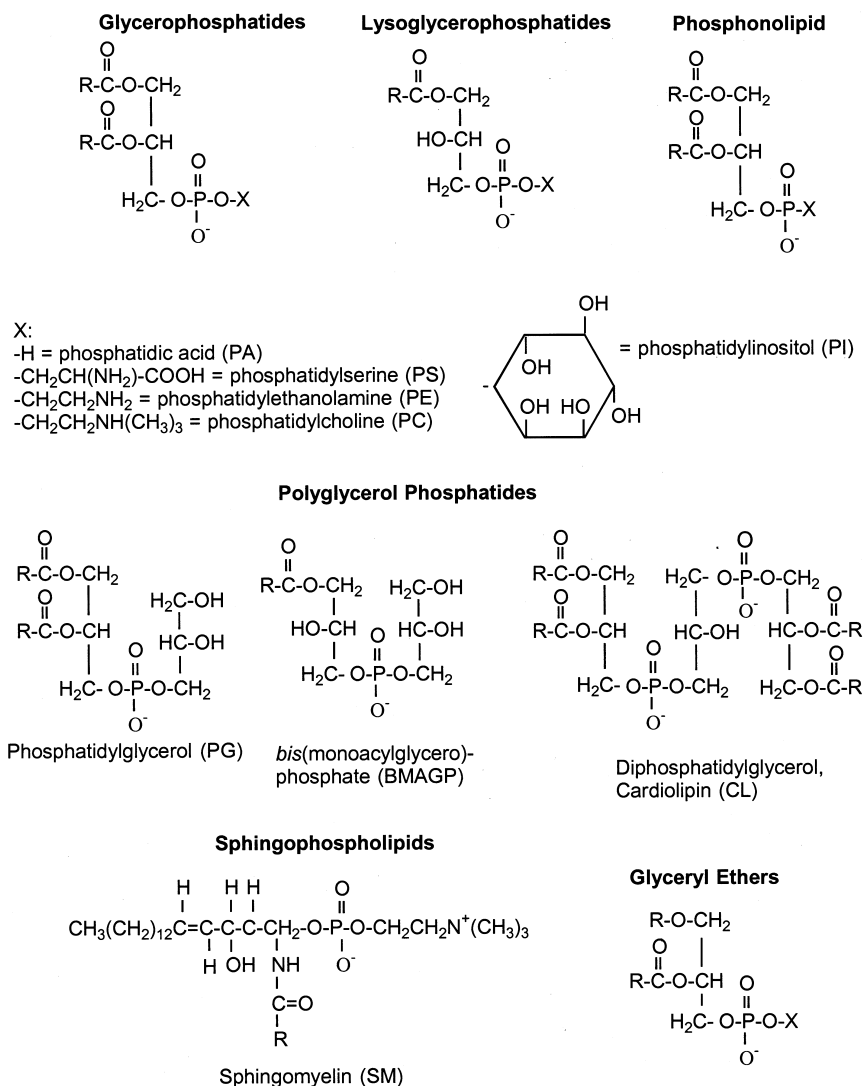


Figure 1 Structures of some complex lipids identified in *Pneumocystis* or mammalian lung.

can lead to changes in the nature of the population, such as its virulence and biochemical composition. Hence it is important to also examine organisms comparable to those present in fulminant lung infections.

Lipid analysis of organisms isolated from mammalian lungs is particularly confounded by the abundance of lung surfactant in the alveolar lining fluid. Lung surfactant comprises mainly lipids (80 to 90%), which can be adsorbed by *Pneumocystis* cell surfaces. The lipid composition of lung surfactant is distinct; disaturated phosphatidylcholine (PC), characteristically with two 16-carbon fatty acids with no double bonds (16:0), predominates [7]. PC alone comprises 79% of total phospholipids in lung surfactant from male Sprague-Dawley rats [8]. Substantial amounts of phosphatidylglycerol (PG), phosphatidylinositol (PI), and cholesterol are also present in mammalian lung surfactant.

Most lipid studies were done on *P. carinii* isolated from the lungs of corticosteroid-immunosuppressed viral antibody-negative rats that had been infected by intratracheal inoculation of cryopreserved *P. carinii* [9,10]. The protocol for isolating and purifying the organisms includes the mucolytic sulfhydryl agent glutathione, which disrupts disulfide linkages bridging the surfaces between adjacent organisms and between *P. carinii* and type I alveolar epithelial cells [9]. Other sulfhydryl agents, such as *N*-acetyl-L-cysteine and dithiothreitol (DTT), are also effective [6,9]; glutathione was selected because it is a normal component of the lung epithelial fluid, where it is found in relatively high concentrations [11]. The delicate tubular extensions of trophic-stage cell surfaces are preserved during detachment when the lung tissues are processed in a Stomacher apparatus, which has a punching/rolling action simulating muscle contractions in a stomach [9].

P. carinii preparations were shown to be >95 to 100% pure [9,12]. Removal of host cells and host nuclei was assessed by light microscopy with bright-field, phase-contrast, differential interference, and fluorescence optics in conjunction with DAPI and other DNA stains as well as calcein AM, propidium iodide, and other vital stains. Absence of host cells and reduction of lung surfactant profiles and debris were visualized by transmission electron microscopy. Elimination of the lung surfactant marker SP-A and exogenously added sterol were monitored by enzyme-linked immunosorbent assay (ELISA) and gas-liquid chromatography (GLC), respectively. The number of potential contaminations by common fungi and bacteria were estimated by culturing on Sabouraud dextrose and Mueller Hinton agar, respectively. The purity of the preparations did not necessitate stripping the cells with the calcium chelator EGTA, which can have adverse effects on cell surface integrity.

The final *P. carinii* preparations purified from rat lungs were conservatively estimated at 89 to 95% viable by dual-stain live/dead analysis [13,14]. Viability was verified by the high level of cellular ATP in the final organism preparations and by metabolic incorporation of radiolabeled precursor compounds [9]. Specific

lipid classes of interest were also evaluated in the lungs of normal rats (normal controls) and the lungs of corticosteroid-treated, uninoculated rats (immunosuppressed controls). Unless otherwise indicated, studies on *P. carinii* described below were performed on preparations obtained by this protocol.

Only some direct biochemical studies have been done on human-derived *P. jirovecii*. Material used for lipid analysis include (1) cryopreserved *P. jirovecii*-infected lung tissue, (2) organisms isolated from cryopreserved autopsied human lungs (purity not defined), (3) formalin-fixed infected lung tissue, (4) bronchoalveolar lavage fluid (BALF), and (5) organisms isolated from BALF (purity not defined). Human BALF is much more viscous than that recovered from rats. Human BALF require high concentrations of DTT to liquify; hence it is more difficult to purify *P. jirovecii* from these samples. Organisms are characteristically found in tight aggregates within a thick mucoid matrix. Formalin-fixed lungs have proved to be valuable sources of *P. jirovecii* sterols, which retain their structures in the preservative [15]. Most analyses of *P. jirovecii* were done to identify lipids already known to occur in *P. carinii*.

III. Uptake of Exogenous Lipid Precursors

Lipid-soluble substrates are taken up by *P. carinii* much faster than water-soluble compounds [1,2,16,17]. Under short-term in vitro culture conditions 18:1 (oleate) was transported faster than 16:0, 18:0, or 18:2, and the kinetic properties suggest that *P. carinii* has fatty acid transporters [16]. The organism may also be able to scavenge lipids directly from host cells [18]. It was estimated that *P. carinii* takes up fatty acids 5 to 20 times faster than water-soluble amino acids [19]. The initial rates of amino acid uptake were examined under chemically defined conditions, taking into account specific activity of the substrate in the incubation medium [20]. Serine, a precursor of several lipids, was taken up at approximately 450 pmol/mg protein/min. The mechanism of uptake was facilitated diffusion; the same carrier also transported leucine and glutamine into the cell.

A number of fluorescent lipid analogues of fatty acids and the complex lipids, ceramides, and phospholipids were used to microscopically document the uptake and internalization of lipids into the organisms [21]. After incubation of organisms with the fluorescent probe at 2°C (to allow adsorption and insertion of the probe), the organisms were washed and then placed at 37°C for translocation and metabolism of the lipid. Most probes were translocated into internal compartments, including spores within thick-walled cysts (Table 1). Trophic forms, presumably vegetatively reproducing, metabolically active stages, exhibited much greater fluorescence than cystic forms. This indicated that the thick cyst wall retards diffusion of these compounds into the organism. The short-chained free C₆-NBD-fatty acid did not insert into surface membranes, but C₁₂-NBD-fatty acid inserted and was translocated into both trophic and cystic stages.

Table 1 Insertion and Degradation in vitro of Fluorescent Lipid Analogues by *Pneumocystis carinii*^a

Fluorescent Lipid Analogue	Insertion of Lipid Analogue	Percent Degraded ^b
C ₆ -NBD-PA	+	50
C ₆ -NBD-PC	+	59
C ₆ -NBD-PE	+	40
C ₆ -NBD-PG	+	52
C ₆ -NBD-ceramide	+	66
C ₆ -NBD-fatty acid	—	
C ₁₂ -NBD-fatty acid	+	

^a Insertion of the probe was performed by incubation at 2 °C for 30 min. After removal of noninserted lipid, translocation and metabolism were allowed to progress at 37 °C for 1 hr.

^b After incubation at 37 °C for 1 hr. Analysis by thin-layer chromatography indicated that approximately half of the fluorescence was in the free fatty acid fraction.

Sources: Refs. 1 and 21.

The metabolic fates of the fluorescent lipids were evaluated 1 hr after incubation; lipids were extracted from cells and wash solutions and analyzed by thin-layer chromatography (TLC) [21]. Incorporation of C₁₂-NBD fatty acid into phospholipids and the formation of new phospholipids from this fluorescent compound were not detected. This indicated that metabolism of these fatty acid, phospholipid, and ceramide analogues was low under the 1-hr incubation conditions used. In contrast, the fluorescent fatty acid analogue Bodipy-C₁₂ was incorporated into *P. carinii* phospholipids and several components in the neutral lipid fraction [18]. Fluorescent fatty acids were present in the wash solution of organisms that had been incubated with fluorescent phospholipids [21]. About half of these fluorescent fatty acids were hydrolyzed from the phospholipid parent compounds after 1 hr of incubation, indicating potent phospholipase activity (Table 1). The localization of the phospholipase A activity responsible for this degradation has yet to be identified.

IV. Lipid Composition of *Pneumocystis carinii*

The profiles of *P. carinii* major lipid classes and those of whole lungs from normal, untreated rats and from corticosteroid-treated, uninfected rats were grossly similar (Fig. 1) (Table 2). In all three groups, the polar lipid fraction was 60 to 80% greater than the neutral lipid fraction. All polar lipids are compatible with membrane bilayers, but the neutral lipid fraction consists of some nonmem-

Table 2 Lipid Class Distributions in *Pneumocystis carinii* and in Rat Lungs—Weight Percent of Total Extracted Lipids

Lipid class	<i>Pneumocystis carinii</i>	Lungs from normal rats	Lungs from immunosuppressed, uninfected rats
Neutral lipid fraction	37.5	36.3	37.9
Free fatty acids	8.2	4.9	4.9
Free fatty alcohols	0.2	0.2	0.2
Monoglycerides	0.2	ND ^a	0.0
Diglycerides	1.1	0.2	0.2
Triglycerides	5.0	18.5	14.3
Free sterols	16.4	9.7	11.0
SteryI esters	6.4	2.7	7.1
Quinones	0.1	0.1	0.2
Polar lipid fraction	62.5	63.7	62.1
Phosphatidylcholine	18.6	23.8	24.1
Lysophosphatidylcholine	1.4	0.5	0.6
Phosphatidylethanolamine	12.6	18.9	11.9
Phosphatidylinositol	9.4	3.1	6.5
Phosphatidylserine	8.0	2.9	1.8
Phosphatidylglycerol	6.3	8.1	6.1
Sphingomyelin	2.1	2.6	5.6
Cardiolipin	1.8	1.7	1.7
Others	2.3	1.9	3.9

^a Not detected.

Sources: Calculated by class compositions within the neutral and polar lipid fractions from data in Refs. 22 and 28.

brane lipids (mono-, di-, and triglycerides; steryl esters; free fatty acids and free fatty alcohols). These normally serve as storage reserves or intermediates in lipid synthesis and catabolism. The level of free fatty acids in *P. carinii* was unusually high [22]. These compounds have surfactant properties and high amounts of them in the cell can be toxic. The relatively high levels of free fatty acids together with the relatively low levels of triglycerides might indicate that fatty acids are significant carbon sources for energy production in *P. carinii*. Rat lungs had higher proportions of triglycerides, containing fatty acid profiles distinct from that of *P. carinii* triglycerides (e.g., 18:2 in the lungs of normal and immunosuppressed uninfected rats were 18 and 11%, respectively, whereas they comprised only 5% of *P. carinii* triglyceride fatty acids). Thus, if the organism scavenged substantial amounts of these lipids, they were apparently rapidly degraded, producing free fatty acids [23]. It was reported that [¹⁴C]palmitate stimulated O₂ consumption,

and $^{14}\text{CO}_2$ was produced by the organism [24,25]. Additional fatty acids and other compounds that may be utilized by *P. carinii* for CO_2 production need to be evaluated by radiorespirometry and compared. This kind of experiment would provide important information on carbon flow and ATP production in *P. carinii*.

Five glycolipids were detected in *P. carinii* lipids by TLC analysis, whereas only four were found in both types of rat lung controls (Z. Amit and E.S. Kaneshiro, unpublished). Thus it appears that the organism has at least one distinct glycolipid component not present in rat lung controls. The structures of these minor lipids were not elucidated. It is likely that additional water-soluble glycolipids are present in *P. carinii*, and that these were not isolated because they partitioned into the aqueous rather than the organic phase during the purification procedure.

Intermediates in sphingolipid synthesis, such as ceramides, are known as potent bioactive molecules in a number of organisms. They can have inflammatory or cytotoxic effects and modulate signal transduction events. Sphingolipids are composed of an amide-linked fatty acid and a long chain base (LCB) (Fig. 1), both of which insert into membrane bilayers. Biosynthesis of LCB occurs by the condensation of a fatty acid with the amino acid serine. While ceramides and LCB were detected in *P. carinii* [19], they have yet to be quantified with respect to the other lipid classes, and more structural data remain to be obtained. Biosynthesis of these lipids was studied in *P. carinii* by metabolic incorporation experiments using radiolabeled serine [19].

Serine was incorporated into both LCB and ceramides after incubation of intact cells in vitro for 18 to 20 hr. However, only trace levels of activity were found in sphingomyelin (SM), suggesting that only low levels of SM, if any, are synthesized by the pathogen immediately after removal from the rat lungs [19]. The apparent absence of SM synthesis is supported by the observation that the organism readily took up fluorescently labeled ceramide in vitro (Table 1) but did not incorporate it into SM [21]. Phytosphingosine and dihydrosphingosine were identified in the *P. carinii* ceramide fraction. Two additional LCBs were detected in this fraction, but their structures remain unknown [19]. These four LCBs were metabolically radiolabeled with serine, indicating that *P. carinii* synthesized them. The serine analogues L-cycloserine and β -Cl-D-alanine inhibited the incorporation of serine into *P. carinii* LCBs and ceramides [19]. D-Cycloserine had no effect, indicating a precise stereochemical specificity of the condensation reaction in *P. carinii* LCB synthesis. The complex sphingolipids—e.g., glycosphingolipids—have not been examined in *P. carinii*; the gangliosides GM_1 and GD_{1a} were identified in *P. jirovecii* [26].

Of the polar lipids, PC was the highest in *P. carinii* [27,28], which reflects lung surfactant phospholipid composition. This apparently results from the uptake of intact complex lipid molecules by the organism [21]. The acidic lipids PI and phosphatidylserine (PS) were present in higher proportions in the *P. carinii*

phospholipid fraction than those of lung tissue (Table 2). Corticosteroid treatment resulted in increased PI in rat lung [28]. Detection of cardiolipin (CL) (Fig. 1), which is restricted to the inner mitochondrial membrane in eukaryotes, is consistent with the presence of well-developed mitochondrial cristae seen in electron micrographs of *P. carinii*. If present, phosphonolipids [phospholipid analogues with direct C-P bond (Fig. 1)] are present in *P. carinii* at only low or undetectable levels, comprising no more than 1% of total phospholipids [28].

P. carinii has the capacity to synthesize in vitro the polar head group of PI [19] as well as PS, phosphatidylethanolamine (PE), and PC but probably not SM. Radioactivity from serine was readily incorporated into PS and PE (by decarboxylation of PS), and much lower radioactivity was found in PC (by methylation of PE). Radioactivity from ethanolamine was incorporated into PE [19,29], with low levels of radioactivity detected in PC [19]. Synthesis of PC may occur at only low rates in *P. carinii*, since this lipid is readily available in lung surfactant.

Glycerol ethers (Fig. 1) were found in *P. carinii* and rat lung controls (Table 3) [30]. Alkyl glycerolipids contain fatty alcohols linked to the glycerol backbone via highly stable C-O-C bonds. The fatty alcohol moiety participates in hydrophobic interactions with other long-chain hydrocarbons such as fatty acids and LCBs within the membrane bilayer. About 3 to 6% of the polar lipids of *P. carinii* and rat lung controls contained ether bonds. All major phospholipid classes contained alkyl glycerolipids in *P. carinii* and both groups of rat lung controls. 1-O-octadecyl glycerol (batyl alcohol), 1-O-octadec-9-enyl glycerol (selachyl alcohol), 1-O-hexadecyl glycerol (chimyl alcohol), and 1-O-hexadec-9-enyl glycerol were identified in all the major phospholipid classes of *P. carinii*

Table 3 Glycerol Ethers of *P. carinii* and Rat Lungs (mol %)

Lipid	<i>Pneumocystis carinii</i> (S/U ^a)	Lungs from normal rats (S/U)	Lungs from immunosuppressed, uninfected rats (S/U)
Total polar lipids	4.8 (4.42)	3.7 (4.85)	5.6 (4.91)
PC	5.4 (6.64)	1.6 (12.24)	2.1 (6.00)
lysoPC	1.4 (6.97)	1.5 (1.53)	2.5 (3.25)
PE	5.0 (9.21)	6.0 (10.81)	13.0 (10.67)
PI	30.6 (4.76)	12.1 (8.26)	45.4 (11.63)
PS	7.5 (4.07)	12.0 (7.76)	10.1 (8.86)
PG	5.6 (4.98)	2.9 (7.14)	4.8 (8.40)

Key: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol.

^a Sum of saturated fatty alcohols/sum of unsaturated fatty alcohols.

Source: Ref. 30.

and rat lung controls. In all cases, chimyl alcohol was the highest. Interestingly, methylprednisolone treatment increased the proportion of alkyl acyl PI in rat lung phospholipids from 12 to 45%. As PI concentration in lung phospholipids was elevated by corticosteroid treatment, the increase in alkyl acyl PI was substantial.

Since all free fatty alcohols in *P. carinii* and rat lung controls were saturated, it was concluded that the formation of the double bond in 1-O-octadec-9-enyl glycerol and in 1-O-hexadec-9-enyl glycerol occurred after the respective alkyl acyl phospholipids were synthesized. Platelet activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) [30] and plasmalogens (alkenyl ethers) [30,31] were not detected in *P. carinii*.

V. Sterols

Of the *P. carinii* lipids, the organism's sterols have received the most attention, mainly because most antimycotics used clinically today target membrane sterols or are sterol biosynthesis inhibitors (SBIs). Polyene antibiotics such as amphotericin B bind to membrane sterols and form large pores that lead to increased permeability and the dissipation of the transmembrane potential. The polyene compounds have higher affinity for ergosterol than cholesterol, and this differential causes the disruption of fungal membranes and not those of the mammalian host. But at higher concentrations, complexes with cholesterol do form. Organisms treated with filipin or digitonin (a detergent that complexes with free β -hydroxysterols) were visualized as intramembranous particles in *P. carinii* membranes by freeze-fracture electron microscopy [32,33]. The membranes of trophic forms had more particles than cystic forms, indicating higher sterol concentrations in the membranes of trophic stages.

A. Sterol Composition: Scavenged and Synthesized

An important feature of the *P. carinii* sterols is the absence of ergosterol (24-methylcholesta-5,7,22-triene-3 β -ol), which is unlike most fungi [34–39]. The organism has no $\Delta^{5,7}$ sterols [36,37,39]. Cholesterol was by far the dominant sterol in *Pneumocystis* (Fig. 2A), consistent with the inefficacy of amphotericin B treatment against *Pneumocystis* pneumonia (PcP). It comprised about 75% of total sterols (see legend to Fig. 2 for the concentrations of major sterols in *P. carinii*). In developing drugs against eukaryotic pathogens, C-24 alkylation and C-22 desaturation are two particularly attractive drug targets [39]. As mammals are incapable of making these sterol modifications, drugs that inhibit these reactions are expected to have low toxicity (cholesterol has a double bond only at C-5 of the sterol nucleus and no alkyl group at C-24 of the sterol side chain). Most fungal lipids are characterized by a methyl or methylene function at C-24 of the sterol side chain. In contrast, plants can form C₂₈ 24-alkylsterols plus those

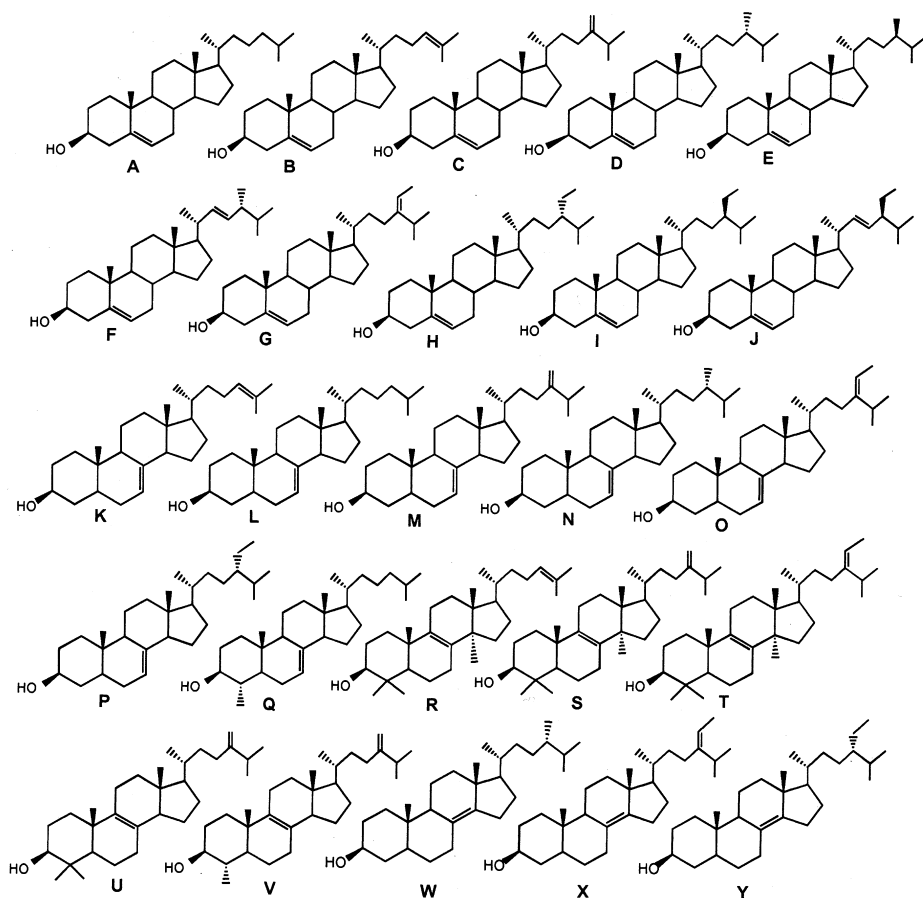


Figure 2 Structures of some sterols identified in *Pneumocystis carinii*. Percent of total sterols is indicated in parenthesis. A-J, Δ^5 sterols; K-P, the major Δ^7 sterols; Q-W, some 4,4-dimethyl- and 4 α -methyl sterols. A. Cholesterol (81.16%). B. Desmosterol (0.35%). C. Ergosta-5,24(28)-dien-3 β -ol (0.65%). D. (24*S*)-Ergost-5-en-3 β -ol (1.92%). E. Campsterol (0.96%). F. Brassicasterol (0.04%). G. Isofucosterol (0.68%). H. (24*S*)-Stigmast-5-en-3 β -ol (0.35%). I. β -sitosterol (1.38%). J. Stigmasterol (0.05%). K. Cholesta-7,24-dien-3 β -ol (0.34%). L. Cholest-7-en-3 β -ol (lathosterol, 0.29%). M. Ergosta-7,24(28)-dien-3 β -ol (1.16%). N. (24*S*)-Ergost-7-en-3 β -ol (fungisterol, 2.51%). O. (Z)-Stigmasta-7,24(28)-dien-3 β -ol (2.60%). P. (24*S*)-Stigmast-7-en-3 β -ol (1.30%). Q. 4 α -Methylcholest-7-en-3 β -ol (0.09%). R. Lanosterol (0.40%). S. 24-Methylenelanost-8-en-3 β -ol (0.29%). T. (Z)-24-Ethylidenelanost-8-en-3 β -ol (pneumocysterol, 0.31%). U. 4,4-Dimethylergosta-8,24(28)-dien-3 β -ol (0.21%). V. 4 α -Methylergosta-8,24(28)-dien-3 β -ol (0.27%). W. (24*S*)-Ergost-8(14)-en-3 β -ol (0.48%). X. (Z)-Stigmasta-8(14),24(28)-dien-3 β -ol (0.29%). Y. (24*S*)-Stigmasta-8(14)-dien-3 β -ol (0.24%).

with ethyl or ethylidene groups at C-24 (C₂₈ and C₂₉ sterols). C-24 Alkylation is catalyzed by *S*-adenosyl-L-methionine:sterol C-24 methyl transferase (SAM:SMT). Like cholesterol, these fungal and plant 24-alkylsterols are characterized by a double bond at C-5 of the sterol nucleus. It is noteworthy that these molecules (C₂₈ and C₂₉ 24-alkylsterols), including ergosterol, are synthesized by several *Leishmania* species and by *Trypanosoma cruzi* (causative agent of Chagas' disease). As in fungi and some plants, these kinetoplastid, flagellated parasites can form double bonds at C-5, C-7, and C-22; the efficacy of SBIs against these parasites is being investigated [40].

The structural identities of 43 sterol molecular species in *P. carinii* were recently elucidated by nuclear magnetic resonance spectroscopy (NMR) [37]; most of these are shown in Fig. 2. The powerful analytical technique of NMR can provide definitive structural information, including stereochemical configurations. However, it is not as sensitive as other techniques, such as gas-liquid chromatography (GLC), requiring larger sample sizes. By pooling many *P. carinii* sterol samples, sufficient material was obtained to make possible the separation of sterols by TLC and high-performance liquid chromatography (HPLC) followed by NMR analysis. Sterol metabolism and biosynthetic pathways in *P. carinii* have yet to be experimentally elucidated; however, the main routes to sterol production in *P. carinii* can be extrapolated based on the data now available (Fig. 3).

Low amounts of plant-derived phytosterols were detected in both *P. carinii* [34,35,37] and rat lung controls [34]. The plant 24-alkylsterols campesterol (24-ergost-5-en-3 β -ol) (Fig. 2E), brassicasterol (24-ergosta-5,22-dien-3 β -ol) (Fig. 2F), β -sitosterol (24-stigmast-5-en-3 β -ol) (Fig. 2I), and stigmasterol (24-stigmasta-5,22-dien-3 β -ol) (Fig. 2J) probably originated from rat food. After uptake and incorporation into the rat [41], these sterols and cholesterol were subsequently taken up and incorporated unchanged into *P. carinii* membranes or converted to sterol esters and stored as such. Stigmasterol and brassicasterol were the only Δ^{22} compounds in the organism, and they comprised only 0.05 and 0.04%, respectively, of total sterols [37]. Thus it is highly unlikely that *P. carinii* desaturates C-22, and this process in sterol metabolism does not represent a drug target in this pathogen.

Although not experimentally ruled out, there is currently no evidence that *Pneumocystis* desaturates C-5 of the sterol nucleus (Fig. 3). The Δ^5 sterols in the organism (Fig. 2A to J) were probably molecules initially scavenged from the host and then remodeled by the organism's SAM:SMT. Of the 13 Δ^5 sterols detected in *P. carinii*, all had the cholesterol nuclear structure and 10 had an alkyl group at C-24 [37]. Preliminary results indicate that Δ^5 sterols are productive substrates for the recombinant *P. carinii* SAM:SMT expressed in bacteria [42] (see below).

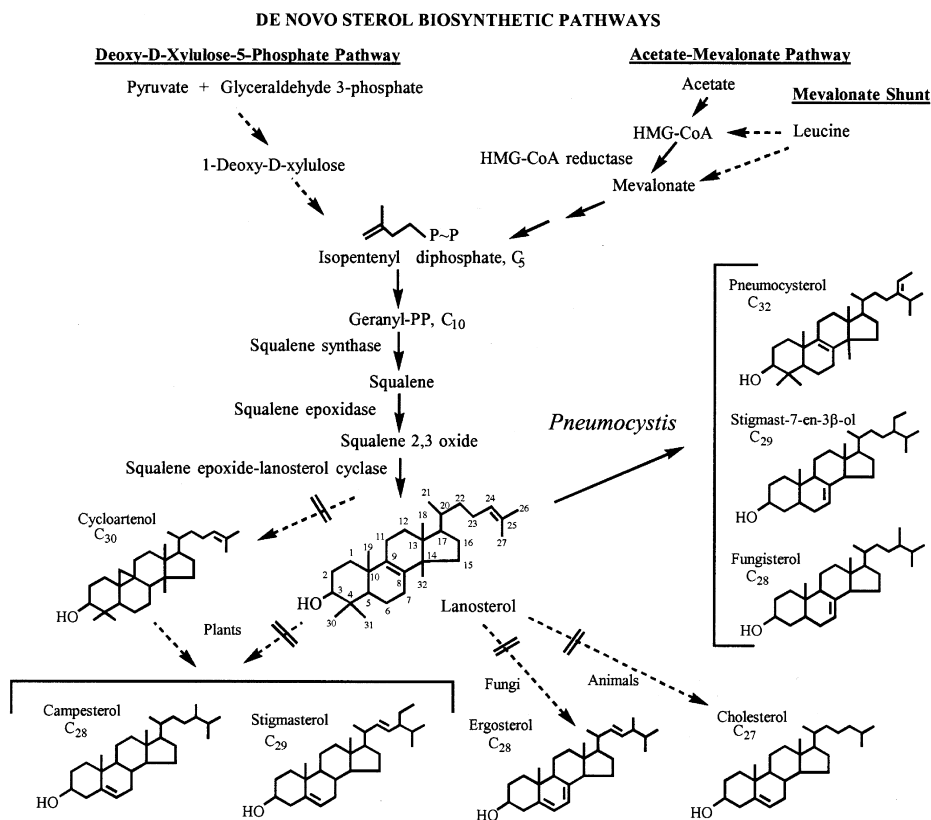


Figure 3 Postulated major sterol biosynthetic pathways in *Pneumocystis*. Solid lines indicate there is evidence for the pathway, broken lines indicate that the existence of the pathway in *P. carinii* is possible or has not been ruled out, two parallel lines indicate no evidence for the pathway in the organism.

B. Sterol Biosynthetic Pathways

That de novo sterol synthesis occurs in *P. carinii* was first suggested by the presence of distinct sterols in the organism that were absent in the rat lung [34,43]. It was then found that the sterol precursors acetate, HMG-CoA, mevalonate, and squalene were metabolically incorporated into *P. carinii* sterols [43,44]. Lovastatin-sensitive HMG-CoA reductase activity was detected ($IC_{50} = 4$ nM) with undetectable or only trace enzyme activity in lung controls [44]. Also, several SBIs had cidal effects on *P. carinii* in vitro [36,45,46] (see below). Furthermore, homologues of genes for the sterol biosynthesis enzymes squalene synthase (*erg9*),

squalene epoxidase (*erg1*), squalene epoxide-lanosterol cyclase (*erg7*), SAM: SMT (*erg6*), and C-24(28) reductase (*erg4*) were identified in the *Pneumocystis* Genome Project by expressed sequence tag (EST) techniques [47]. Thus, despite scavenging the bulk of its sterols from the alveolar environment, the organism synthesizes its own distinct sterol molecules. De novo sterol biosynthetic pathways in *P. carinii* (Fig. 3) apparently lead to the formation of the C₂₈ and C₂₉ Δ^7 alkylsterols (Fig. 2K to Q) that dominate the organism-specific sterols. With the exception of the intracellular amastigote life-cycle stage of *T. cruzi* [48], these are unusual molecules not normally found in other animal pathogens. The only other organisms known to accumulate high levels of these lipids are the rust fungi; this is also a unique feature of these basidiomycete plant pathogens [49–51].

Probably most or all the Δ^8 sterols (Fig. 2Q to W) are likewise synthesized de novo by *P. carinii*. Lanosterol (Fig. 2R), a product of squalene cyclization, is a common precursor in most organisms; cycloartenol is its homologue in some plants. Lanosterol was detected in *P. carinii*, but cycloartenol was not detected by both GLC-mass spectrometry (MS) and NMR analyses [37,52]. Also, the cloned *P. carinii erg7* gene expressed in a *S. cerevisiae* lanosterol synthase null mutant complemented the sterol deficiency [53]. The *P. carinii erg7* oxidosqualene cyclase gene coded for the enzyme that produces lanosterol, not cycloartenol.

The C-24-alkylated lanosterol derivative 24(Z)-ethylidinelanost-8,24(28)-3 β -ol (Fig. 2S) was detected in low amounts in fungi and protozoa inhibited by various compounds [54]. It was also identified as a minor component of normal, untreated *Neolitsea sericea* [55], a lauracean plant commonly known as the pencil cactus. This rare C₃₂ sterol was given the trivial name pneumocysterol when large amounts were found in *P. jirovecii* [16,56,57]. Pneumocysterol comprised about half of the organism-specific sterols in some *P. jirovecii* samples. In contrast, only low or trace amounts were found in some other *P. jirovecii* samples [57], *P. carinii* [37], and samples of *Pneumocystis*-infected lungs from corticosteroid-immunosuppressed mice and ferrets, mice, with severe combined immune deficiency (SCID), weanling rabbits, and monkeys infected with simian immunodeficiency virus (SIV) [58]. Among the *P. jirovecii* samples, pneumocysterol level was not correlated with HIV infection or drug therapy. In a study of samples taken from different sites of a single individual's lungs, a broad range of pneumocysterol values were observed, ruling out HIV infection or drug effects as the basis for the quantitative difference in pneumocysterol concentration [58,59]. This observation suggested that there are at least two *P. jirovecii* genetic populations that differ in the regulation of sterol biosynthesis and hence in pneumocysterol accumulation [59].

C. Sterol Biosynthesis Inhibitors (SBIs)

Several SBIs were shown to have cidal effects on *P. carinii* in vitro; cidal effect was defined as $\geq 50\%$ reduction in *P. carinii* cellular ATP levels after 1 to 3 days

of exposure [46] (Table 4). All HMG-CoA reductase inhibitors tested (lovastatin; simvastatin; L-647,318; L-654,164) had no effect on ATP levels. This suggests that the organism might also produce isoprenoids via another pathway or pathways in addition to that involving the reaction of HMG-CoA to mevalonate (Fig. 3). While there is no evidence in *P. carinii* for the deoxy-D-xylulose pathway, preliminary results indicate that there may be a pathway from leucine to mevalonate [60]. The presence of an additional pathway involving reactions that allow bypassing HMG-CoA is consistent with the low HMG-CoA reductase activity in *P. carinii* (0.1 nmol/mg protein/min) compared to other organisms (e.g., 130 nmol/mg total rat liver homogenate protein/min) [61]. Also, incubating *P. carinii* cells with radioactive mevalonate in the presence of lovastatin (to block de novo synthesis of nonradioactive mevalonate via HMG-CoA) did not enhance radiolabeling of sterols and ubiquinones [62]. The existence of a pathway bypassing HMG-CoA could explain this observation.

The squalene synthase inhibitors CCI 14993 and CCI 16543 had anti-*P. carinii* activity, whereas squalenstatin did not [46]. The squalene epoxidase inhibitor tolnaftate had only a slight effect, but terbinafine effectively reduced ATP levels, which was especially evident after 72 hr. Some in vivo studies, using corticosteroid-immunosuppressed rats or mice, are in agreement with the terbinafine in vitro results [45], whereas others are not [63]. All squalene epoxidase-lanosterol cyclase inhibitors tested (GR 90525A, GR 193018A, GR 54985A, UI 9666A, GR 31149A) reduced *P. carinii* ATP [46]. Since parasites can scavenge preformed molecules such as sterols from the host, a single block at a step early in the synthesis of compounds essential to the pathogen may not be as effective in vivo as a block just prior to the formation of the *Pneumocystis*-specific sterol molecules. Inhibitors that block early steps in combination with those that block late steps may be a good strategy for getting additive or synergistic effects in vivo.

The herbicide AY 9944 inhibits Δ^8 to Δ^7 isomerization. This compound was cidal to *P. carinii* in vitro, thus identifying another potential attractive drug target in the organism [46]. All but one Δ^7 sterol molecule detected in the organism lacked methyl groups at C-4 and C-14 (Fig. 2K to Q) [37], suggesting that, in most cases, this shift in double-bond position occurred after sterol nucleus demethylation. Four other C-24 alkylsterols with an 8(14) double bond also lacked C-4 and C-14 methyl groups (Fig. 2W to Y) [37]; they may represent intermediates in Δ^8 to Δ^7 isomerization.

Interestingly, while the lanosterol demethylase inhibitors GR 40665X, GR 40317A, and GR 42539X had potent effects, fluconazole, GR 71539X, and GR 77303X had no effect [46]. This suggests that the latter three, which are effective against other fungal pathogens such as *Candida*, may not inhibit sterol nuclear demethylase enzymes in *P. carinii* because the substrate preferences of *P. carinii* enzymes differ from those of most fungi. This suggestion is supported by the

Table 4 Effects of Sterol Biosynthesis Inhibitors on *P. carinii* ATP Content—IC₅₀ (μM) at Three Different Exposure Times

SBI Target	24 hr	48 hr	72 hr
HMG-CoA reductase			
Simvastatin	— ^a	—	2422
Lovastatin	—	—	—
L-647,318	—	—	—
L-654,164	—	—	—
Squalene synthase			
CCI 14993	2832	18	8
CCI 16543	434	68	33
Squalestatin (GR 105155X)	—	—	—
Squalene epoxidase			
Terbinafine	5863	130	13
Tolnaftate	—	3980	1625
Squalene epoxide-lanosterol cyclase			
GR 90525A	7	4	3
GR 193018A	30	11	8
GR 54985A	121	16	9
UI 8666A	212	60	67
GR 31149A	4464	47	6
Lanosterol demethylase			
GR 40317A	33	8	5
GR 42539X	81	8	7
GR 40665X	692	453	372
Fluconazole	—	—	—
GR 71539X	—	—	—
GR 77303X	—	—	—
Δ ⁸ to Δ ⁷ isomerase			
AY 9944	51	36	7
SAM:SMT			
24(25)-Epiminolanosterol	—	4737	—
24-Bromolanosterol	—	—	—
24-Iodolanosterol	—	—	—
Sinefungin	—	—	—

^a Inhibition was always less than 50%.
Source: Ref. 46.

recent finding that lanosterol is the preferred substrate for the recombinant *P. carinii* SAM:SMT expressed in *Escherichia coli* [64]. The SAM:SMTs from other fungal and plant sources normally prefer sterol substrates that have undergone nuclear demethylation, (e.g., zymosterol) and do not productively bind lanosterol.

24-Alkylsterols are formed by the transfer of a methyl group from SAM to a sterol substrate by the enzyme SAM:SMT. *Pneumocystis* requires exogenous SAM and rapidly takes up this compound from the axenic culture medium [65]. When SAM:SMT activity in *P. carinii* was blocked by 20-piperidin-2-yl-5 α -pregnan-3 β -20(R)-diol or 24(25)-epiminolanosterol, 24-alkylsterols in the organism decreased and organism proliferation in vitro was inhibited [36]. The *P. carinii* SAM:SMT is therefore an attractive drug target in this pathogen. However, 24(25)-epiminolanosterol, 24-bromolanosterol, and 24-iodolanosterol had no effect on cellular ATP levels [46], suggesting that SAM:SMT inhibition by these compounds might have static effects, but cidal activity was not evident. The efficacy of these putative SAM:SMT inhibitor compounds has yet to be tested directly on the *P. carinii* enzyme activity. Some steroidal allenic phosphonate derivatives may also inhibit *P. carinii* SAM:SMT [66]. Several compounds, especially 17-[2-(diethylphosphonato)ethylidienyl]3-methoxy-19-norpregna-1,3,5-triene, had cidal effects on *Leishmania donovani* and *L. mexicana* in vitro. After treatment, *Leishmania* culture growth in drug-free medium was inhibited. The effects of these compounds were tested on *P. carinii*; they depleted cellular ATP levels and also decreased organism viability, as shown by live/dead fluorescence staining [66]. These compounds also need to be tested directly on the organism's SAM:SMT enzyme and on *P. carinii* culture growth under drug-free conditions after exposure to the drugs.

A significant advancement in *P. carinii* SAM:SMT research was recently achieved using recombinant protein techniques. There were 11 entries identified in the *Pneumocystis* Genome Project EST database for the *erg6* gene, which codes for SAM:SMT. The *P. carinii* *erg6* gene was sequenced, cloned, and expressed in *E. coli* and the *erg6* null mutant of *Saccharomyces cerevisiae* [67]. The nucleic acid sequence and the deduced amino acid sequences were homologous to those of fungi and plants. While the two putative sterol- and three SAM-binding site motifs were homologous to those of known SAM:SMT, the *P. carinii* SAM:SMT sterol-binding motif was novel, consistent with the unique high-affinity binding of lanosterol [64]. As *E. coli* does not synthesize sterols, exogenous sterol substrates and products are readily identified in bacteria expressing the *P. carinii* *erg6* gene. The SAM:SMT in plants transfers one or two methyl groups; some plants have separate genes apparently encoding distinct enzymes for the first and the second reaction. The *P. carinii* recombinant enzyme in *E. coli* transferred methyl groups from SAM to the C-24 position of both lanosterol and 24-methylenelanosterol [64]; thus the enzyme is capable of transferring both the

first and second methyl groups. The effects of various SBIs on the purified *P. carinii* recombinant SAM:SMT protein have yet to be reported.

There is now good evidence that *P. carinii* can utilize cholesterol for forming the bulk phase of membrane bilayers and produces its own metabolic sterols [40], because these apparently have the precise stereochemical structures required for its membranes to operate properly [34]. In addition to identifying potential drug targets, the elucidation of *P. carinii* sterol structures may prove useful for detecting the organism in environmental and clinical samples. Lipid profiles and distinct lipid molecules specific for an organism or group of organisms are now routinely employed as biomarkers for identifying and characterizing microbial communities [68,69]. The relatively rare and unusual *Pneumocystis* sterol pneumocysterol might be used as a signature for independent validation of certain *Pneumocystis* strains [70].

VI. Ubiquinones

Ubiquinone (coenzyme Q, or CoQ) plays a pivotal role in mitochondrial electron transport. In the inner mitochondrial membrane of eukaryotic cells, CoQ acts as a mobile electron carrier from complexes I and II to the cytochrome *bc*₁ complex (complex III) or the alternative oxidase pathway. During the process of electron transport, CoQ undergoes oxidation-reduction reactions to transfer electrons by occupying two sites in the *bc*₁ complex. At the Q_i site, it accepts electrons that come from dehydrogenase enzyme activities; at the Q_o site, it donates electrons. It is believed that CoQ fits in a pocket of the Q_o domain of the *bc*₁ complex and binds to cytochrome *b*. An electrochemical gradient is maintained across the inner mitochondrial membrane by the translocation of protons from the matrix to the mitochondrial intermembrane space by the electron transport complexes. When electron transport is disrupted, this inhibits a number of dehydrogenase enzyme activities, respiration (oxygen consumption), and ATP production because electrons originating from the dehydrogenase enzymes go through the electron transport chain and are ultimately passed to molecular oxygen. Phosphorylation of ADP to ATP is coupled to this process because the reaction is driven by the flow of protons back into the mitochondrial matrix compartment through ATP synthase in the membrane.

The mechanism of cell surface electron transport has yet to be examined in detail in any cell type [71,72], but the presence and enrichment of ubiquinone in the cell surface membrane was documented by direct biochemical analysis (*Paramecium* ciliary membrane) [73]. Also, chloroquine inhibition of *Tetrahymena* [74] and *Leishmania* promastigote [75] cell surface electron transport and associated proton pumping suggested that this surface membrane function may be an attractive drug target [74–76]. Ubiquinone may play a role in cell surface transmembrane electron and proton translocation in *P. carinii*, perhaps by interact-

ing with the P-type proton ATPase pump. The *P. carinii* *pca1* gene, homologous to the yeast *pma1* gene that codes for a proton pump protein, has been cloned and characterized [77]. The *Pneumocystis* Genome Project includes entries in its EST database indicating that genes homologous to *pma1* have been identified; thus this gene is actively expressed. The *P. carinii* *pca1* gene was able to complement a *Saccharomyces* null mutant lacking homologous pump proteins (Meade, personal communication, 2002). A cell surface proton pump appears to generate a proton gradient and hence proton motive force. There is evidence that this pump is vital to *P. carinii*; it is responsible for maintaining the organism's intracellular pH [78] and the cell surface membrane electrical potential [79]. The *P. carinii* high-affinity glucose transporter is apparently driven by proton motive force; the proton ionophore CCCP and the P-type proton ATPase inhibitor DCCD inhibited uptake of 2-deoxyglucose [80]. Imipramine, which inhibits P-type proton pumps [81], reduced *P. carinii* cellular ATP in vitro [82]. The cell surface proton pump is also an attractive drug target in *P. carinii*, but whether CoQ plays a role in its function needs further investigation. As CoQ levels in the endoplasmic reticulum–Golgi complex are high in some cells, often exceeding those in mitochondria, it is probably here that cell surface CoQ originates.

Ubiquinone is synthesized by condensation of *p*-hydroxybenzoic acid (PHBA) with a polyprenyl diphosphate chain, followed by several modifications of the aromatic ring (Fig. 4). PHBA is a product of chorismate, formed via the shikimate pathway, which is absent in mammals. The *arom* gene, located in the nucleus, was one of the earliest in *P. carinii* to be cloned and sequenced [83]. This multifunctional gene encodes five enzymes in the shikimate pathway. Other vital compounds, such as folic acid and the aromatic amino acids, are also produced from chorismate. The detection of the *arom* gene suggested that *P. carinii* synthesizes de novo the benzoquinone moiety of CoQ. Inhibition of *P. carinii* axenic culture proliferation by glyphosate, a specific inhibitor of the shikimate pathway, is consistent with chorismate synthesis de novo [84].

Total ubiquinone concentration in *P. carinii* was higher than that in rat lungs (22 and 9 pmol/mg protein, respectively), but both were much lower than that found in other cell types such as rat liver (121 pmol/mg protein) and rat heart (284 pmol/mg protein) [22]. CoQ₁₀ was identified as the major homologue in *P. carinii*, and CoQ₉ was also detected. In contrast, CoQ₉ was the dominant homologue in rat lung controls and CoQ₁₀ was not detected, suggesting that at least CoQ₁₀ was made by *P. carinii*. Subsequently, sensitive metabolic radiolabeling experiments showed that *P. carinii* synthesizes de novo CoQ₇, CoQ₈, CoQ₉, and CoQ₁₀ [62]. The benzoquinone precursors shikimate, PHBA, and tyrosine and the polyprenyl chain precursors mevalonate and several polyprenyl diphosphates were incorporated into the organism's ubiquinones [22,62] (E.S. Kaneshiro and M. Basselin, unpublished). Incorporation of radiolabeled PHBA into CoQ was not detected in homogenates of both types of rat lung controls.

Shikimate Pathway

Isoprenoid Pathway

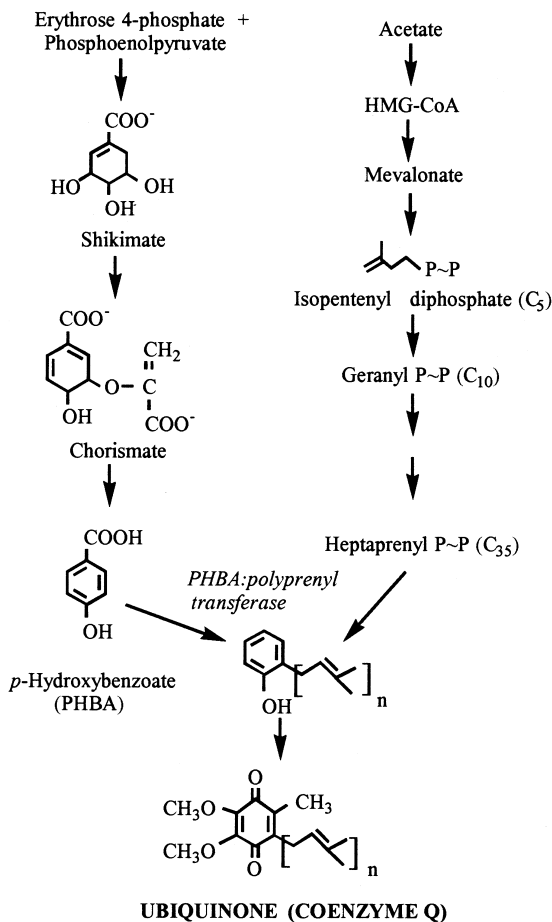


Figure 4 Generalized schematic of de novo ubiquinone biosynthesis. The benzoquinone moiety is derived from chorismate, the product of the shikimic acid pathway that leads to the formation of folic acid, aromatic amino acids, and other compounds. The polyprenyl chain is derived from the same isoprenoid synthesis pathways that lead to the formation of sterols and isoprenylated proteins.

The anti-*Pneumocystis* pneumonia drug atovaquone (Fig. 5A) [85–88] is an analogue of CoQ, and it effectively blocks mitochondrial electron transport by high-affinity binding to cytochrome *b* of the *bc*₁ complex [86] (see also Chap. 24). As a consequence, ATP levels in *P. carinii* are depleted [89]. In addition to blocking electron transport, it was found that atovaquone also inhibited *P. carinii* CoQ biosynthesis [90,91]. Interestingly, stigmatellin (Fig. 5C), which apparently also binds to the *bc*₁ complex by similar stereochemical interactions, did not affect *P. carinii* CoQ synthesis [91]. This observation suggested that there are important differences between drug binding to the *bc*₁ complex and in the CoQ biosynthetic machinery. Atovaquone (10 nM) reduced CoQ synthesis in intact cells by 60% [91]. Using whole cell homogenates, the mechanism of drug action was shown to be by competitive inhibition, but the maximal inhibition was also limited to 60% [91,92]. Several other naturally occurring and synthetic ubiquinone analogues also inhibited the incorporation of PHBA into *P. carinii* CoQ; the most effective was bupravaquone (Fig. 5B). Inhibition of CoQ synthesis by all compounds did not exceed 60%. The inability to detect complete inhibition was subsequently explained by subcellular fractionation of *P. carinii*, which enabled dissection of CoQ synthesis into the mitochondria and the endoplasmic reticulum (ER)–Golgi compartments. Both the mitochondrial and microsomal

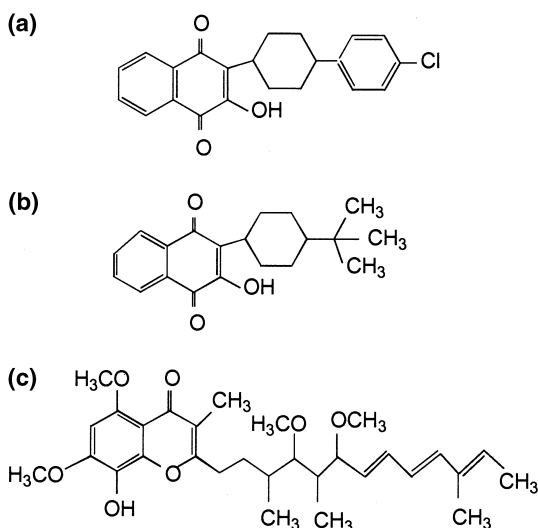


Figure 5 Inhibition of PHBA incorporation in *P. carinii* ubiquinones. Atovaquone (A) and bupravaquone (B) were good inhibitors, whereas stigmatellin (C), which binds to mitochondrial cytochrome *bc*₁ complex by similar stereochemical interactions as atovaquone, had no effect on *P. carinii* ubiquinone biosynthesis.

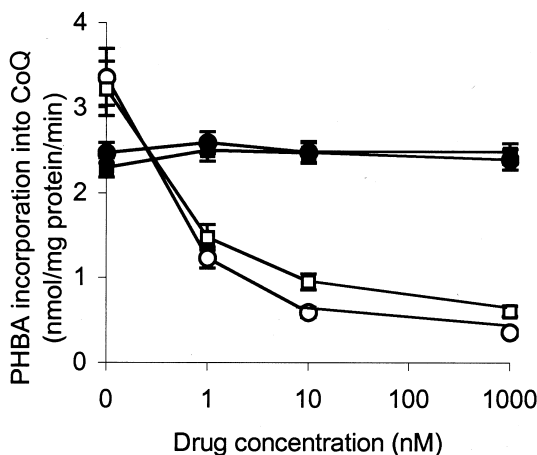


Figure 6 Inhibition of PHBA incorporation into ubiquinones in *P. carinii* subcellular fractions. Solid symbols, mitochondrial fraction; open symbols, microsomal fraction; squares, atovaquone; circles, bupravaquone.

fractions synthesized all four CoQ homologues. Atovaquone inhibited CoQ synthesis in the microsomal fraction, and the syntheses of all homologues were inhibited by atovaquone and bupravaquone. However, CoQ synthesis in the mitochondrial fraction was not affected by either of these drugs (Fig. 6) [91,92]. Thus, CoQ₇, CoQ₈, CoQ₉, and CoQ₁₀ are synthesized de novo in both the ER-Golgi complex and the mitochondrion, but the two cell compartments exhibited differential responses to the CoQ analogues atovaquone and bupravaquone. These drugs inhibited ER-Golgi but not mitochondrial CoQ synthesis in this organism (Fig. 6) [91,92] (E.S. Kaneshiro, M. Basselin and S.M. Hunt, unpublished).

VII. Fatty Acids

A. Total Cellular and Total Extracted Lipids

Straight-chain saturated hydrocarbon chains (e.g., fatty acids, fatty alcohols, and LCBs) within membrane bilayers increase order and decrease the fluidity and lateral mobility of membrane components. *Pneumocystis* lives in an environment with abundant lipids comprising mainly saturated fatty acids, which the pathogen can utilize. Lipids with other properties are also required to maintain optimal membrane function. Unsaturated short- and branched-chain fatty acids, those with bulky groups such as a cyclopropane ring, or those containing electronegative atoms serve to loosen the bilayer packing and increase membrane fluidity.

The composition of total cellular fatty acids (in lipids plus those linked to other molecules such as proteins and carbohydrates) [31] and those of total extracted lipids [93,94] grossly reflect that of lung tissues, with 16:0 (palmitate) present in the highest concentrations (Table 5). When organisms were isolated from rat lungs and then cocultured with WI-38 cell monolayers, the percentage of 18:1 in total cellular fatty acids increased, whereas 18:0 and 16:1 decreased from day 1 to day 7 [31]. These changes suggest that *P. carinii* synthesizes de novo high proportions of 18:1. However, these observations can also reflect, at least in part, to the uptake and incorporation of fetal bovine serum fatty acids, which is higher in 18:1 and lower in 16:0 compared to lung surfactant or tissue [22,95]. This interpretation is consistent with other observations that *P. carinii* scavenges much of its lipids from exogenous sources.

Low concentrations of the epoxy fatty acid 9,10-epoxy octadecanoic acid (epoxy 18:0) (Fig. 7) were detected among the *P. carinii* total lipids [22,96,97]; it was not detected in rat lung controls. It is interesting to note that the rust fungi accumulate high concentrations of epoxy fatty acid [98] in addition to C₂₈ and C₂₉ Δ^7 24-alkylsterols (see above). Although it lacks a double bond, the bulky epoxide ring, which is located in the middle of the hydrocarbon chain, would decrease hydrophobic interactions with other bilayer lipid components and increase membrane fluidity. The saturation index (sum of saturated fatty acids/sum of unsaturated fatty acids, S/U) of total lipids, total polar lipids, and individual membrane lipid classes of *P. carinii* is unusually high. Although the organism is maintained at 37°C in the lung, the fatty acid S/U of *P. carinii* membrane lipids is higher than that of most mammalian cells, which generally contain less saturated than unsaturated acids. It is likely that even low concentrations of lipid components such as epoxy 18:0 can play an important role in conferring optimal membrane fluidity in the organism.

9,10-Epoxy octadecanoate in *P. carinii* is synthesized from oleate (Fig. 7) [97]. When organisms were incubated for 24 hr in an axenic culture medium supplemented with 18:1, epoxy 18:0 levels in the organism increased. An epoxigenase enzyme in *P. carinii* catalyzed the formation of epoxy 18:0 from radioactive 18:1 in vitro; activity was optimal at 37°C and pH 7.5 (K. Chin and E.S. Kaneshiro, unpublished).

Analysis of only the fatty acids linked by ester bonds in *P. carinii* indicated that these were enriched in unsaturated acids (Table 5). In contrast, S/U of ester-linked fatty acids of whole lungs from normal, untreated rats and corticosteroid-treated, uninfected rats were >1 (1.09 and 1.27, respectively) [94]. The majority of ester-linked fatty acids are found in glycerophospholipids of the polar lipid fraction and in steryl esters and mono-, di-, and triglycerides in the neutral lipid fraction. The fatty acids attached via amide bonds are found in SM and ceramides; these were almost exclusively saturated molecular species in *P. carinii* and the rat lung controls. Since the fatty acid-containing lipids in the neutral lipid fraction

Table 5 *Pneumocystis carinii* Fatty Acid Composition—Weight Percent

Fatty acid	Total cellular ^a	Total extracted lipids ^b	Total esterlinked ^b	Total amidelinked ^b	Total neutral lipids ^b	Total polar lipids ^c
12:0	ND ^d	ND	ND	ND	1.3	ND
14:0	2.7	1.1	1.7	1.7	2.3	0.2
15:0	ND	0.2	0.4	0.8	1.1	0.3
16:0	40.4	44.5	27.3	40.7	37.3	25.4
16:1	4.7	2.1	3.2	1.5	3.8	2.2
17:0	ND	0.2	0.2	0.9	0.6	0.2
18:0	11.8	13.2	8.4	7.8	10.8	12.2
Epoxy 18:0 ^e	ND	0.5	ND	ND	ND	Trace
18:1	18.5	13.4	23.9	9.0	19.4	32.0
18:2	4.0	1.8	7.0	2.3	2.6	5.7
18:3	ND	ND	ND	ND	0.9	ND
20:0	ND	0.3	0.1	1.4	2.1	0.5
20:1	ND	ND	ND	ND	2.4	ND
20:3	ND	ND	ND	ND	ND	5.7
20:4	5.0	2.4	10.6	1.1	0.6	5.2
20:5	ND	ND	ND	ND	ND	0.3
21:0	ND	0.2	ND	0.7	ND	1.7
22:0	ND	1.7	0.1	4.6	0.5	ND
22:1	ND	0.2	1.4	0.9	2.5	0.9
22:3	ND	ND	ND	ND	1.2	ND
22:4	ND	ND	ND	ND	ND	3.7
24:0	ND	1.9	0.7	12.6	0.8	2.3
24:1	ND	ND	ND	7.0	0.6	2.4
26:0	ND	ND	ND	ND	0.1	ND
28:0	ND	ND	ND	ND	0.1	ND
Others ^f	8.4	16.5	16.7	7.3	8.5	1.9
PUFA ^g	9.7	4.2	17.6	3.4	5.3	20.6
S/U ^h	1.69	3.23	0.82	3.17	1.68	0.74

^a Values are from day 1 of short-term cultures (from Ref. 31).^b From Ref. 22.^c From Ref. 94.^d ND., not detected or not determined.^e Chin K, Kaneshiro ES, unpublished.^f Sum of unidentified fatty acids and/or those present in low concentrations.^g PUFA, sum of polyunsaturated fatty acids.^h Saturation index (sum of saturated acids/sum of unsaturated acids) not including epoxy 18:0.

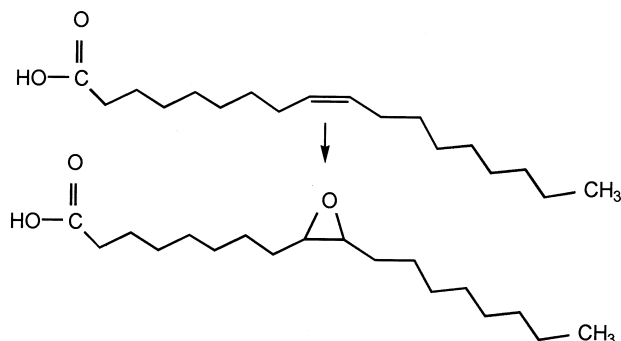


Figure 7 Synthesis of 9,10-epoxy dodecanoic acid from oleic acid in *P. carinii*.

are not membrane components, it is not surprising that the fatty acid profile of this fraction was almost identical to that of lung controls (normal, untreated, and corticosteroid-treated uninfected rats). In contrast, the entire polar lipid fraction is composed of integral membrane compounds, and it is within this group that clear distinctions between organism and host lung were evident. The S/U of the *P. carinii* total polar lipid fraction was <1 (Table 5), whereas S/U was 1.47 and 2.90 in lungs from normal, untreated, and corticosteroid-treated uninfected rats, respectively [28]. There were significantly higher percentages of the unsaturated acids 18:1, 22:4, and 24:1 and lower 16:0 in the *P. carinii* polar lipid fraction compared to both lung controls.

B. Individual Lipid Classes

Fatty acid compositions of individual lipid classes further help in dissecting components that determine the physical properties of the organism's membrane bilayer. With the exception of PE, the fatty acid saturation indices of all other glycerophospholipid classes were >1 (Table 6) [94]. Palmitate was maintained at only moderate levels in *P. carinii* PE, which had the lowest percentage of saturated acids and the highest polyunsaturated acids among all phospholipid classes. Also, PE contained the highest percentage of epoxy 18:0, which suggests that this phospholipid class plays a major role in enhancing the fluidity of *P. carinii* membranes. The epoxy 18:0 was located at the C-2 position of the PE glycerol backbone, as shown by phospholipase A_2 digestion (K. Chin and E.S. Kaneshiro, unpublished). As PE makes up a substantial portion of the bulk of membrane phospholipids (Table 2), the relatively low S/U of both the total polar lipid fraction and the total ester-linked fatty acids of the organism (compared to those of lung controls) can be attributed to a great extent to PE. PE in both types of rat lung controls had the lowest S/U (1.44 in both) among the glycerophospho-

Table 6 Fatty Acid Composition of Individual Lipid Classes in *P. carinii* Organisms

Fatty acid	MG	DG	TG	FFA	SE	PC	LPC	PE	PS	PI	PG	SM	CL
12:0	0.6	1.0	0.4	0.8	0.8	ND	ND	ND	ND	ND	ND	ND	ND
14:0	4.4	2.8	3.1	3.2	3.4	0.9	1.3	3.3	2.3	0.7	3.6	1.2	3.6
15:0	2.3	1.4	1.2	1.4	1.1	0.4	1.3	3.5	5.1	0.9	34.0	0.5	5.3
16:0	40.9	43.5	39.9	56.3	45.4	49.1	48.1	8.0	17.8	17.2	28.1	15.1	14.5
16:1	2.8	2.0	1.6	2.2	2.4	1.4	4.4	2.8	1.9	0.8	1.3	1.0	3.4
17:0	1.0	0.9	0.9	1.5	0.8	0.1	1.0	0.9	8.3	0.6	5.0	0.3	1.7
18:0	17.5	14.9	17.4	18.4	14.2	8.8	14.5	14.4	16.1	26.3	18.0	13.3	18.6
Epoxy 18:0 ^b	ND	ND	ND	ND	ND	Trace	ND	3.0	ND	ND	ND	ND	ND
18:1	12.8	12.2	13.2	6.6	9.5	34.2	6.8	36.7	6.5	27.2	21.7	1.4	9.8
18:2	3.4	4.9	5.1	2.5	5.0	4.9	2.4	5.3	Trace	3.1	4.1	0.6	9.1
18:3	1.1	1.7	2.6	1.1	1.6	ND	ND	ND	ND	ND	ND	ND	ND
19:0	ND	ND	ND	ND	ND	ND	ND	ND	9.0	ND	ND	ND	ND
20:0	0.9	1.0	1.1	1.1	1.2	0.3	1.8	1.8	5.0	1.6	1.5	6.7	1.7
20:1	1.0	2.8	0.8	0.5	1.7	ND	ND	ND	ND	ND	ND	ND	ND
20:3	ND	ND	ND	ND	ND	0.2	0.3	3.6	1.4	0.3	6.7	Trace	6.0
20:4	2.0	2.1	1.9	0.7	2.4	4.0	1.1	1.0	5.7	1.3	4.3	2.9	1.8
20:5	ND	ND	ND	ND	ND	0.6	ND	0.8	0.4	0.6	1.4	0.3	ND
21:0	ND	ND	ND	ND	ND	0.3	1.5	4.6	11.2	2.0	1.6	Trace	3.9
22:0	0.7	0.5	0.3	0.7	0.6	0.4	1.8	1.1	2.0	2.3	1.1	16.7	2.7
22:1	0.2	0.2	0.1	0.2	0.3	Trace	4.1	3.0	7.5	2.1	2.7	0.8	6.7
22:3	0.1	Trace	0.2	0.1	0.4	ND	ND	ND	ND	ND	ND	ND	ND
22:4	ND	ND	ND	ND	ND	Trace	1.1	7.1	Trace	1.2	ND	ND	1.6
22:5	ND	ND	ND	ND	ND	0.6	ND	0.7	Trace	Trace	0.3	ND	ND
23:0	ND	ND	ND	ND	ND	ND	2.3	ND	1.7	2.1	ND	ND	0.3
24:0	1.4	1.4	1.4	0.7	1.9	0.7	1.3	1.1	2.9	Trace	3.2	37.3	0.4
24:1	0.1	Trace	0.1	0.1	0.6	0.4	2.3	1.5	4.6	1.0	0.8	5.4	0.9
26:0	1.2	1.1	1.1	0.3	1.0	ND	ND	ND	ND	ND	ND	ND	ND
28:0	0.2	Trace	ND	ND	ND	Trace	ND	ND	ND	ND	ND	ND	ND
Others ^c	5.5	5.6	7.8	1.4	5.9	2.2	3.7	0.3	0.7	3.4	1.4	2.3	7.2
PUFA ^b	6.6	8.7	9.8	4.4	9.4	10.1	4.6	14.9	6.1	6.2	10.1	3.8	12.5
S/U ^e	3.02	2.64	2.61	6.03	2.95	1.32	3.33	0.62	2.91	1.43	1.53	7.35	1.34

Key: MG, monoglycerides; DG, diglycerides; TG, triglycerides; FFA, free fatty acids; SE, steryl esters; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; CL, cardiolipin.

^a Not detected or not determined.

^b Chin K, Kaneshiro ES, unpublished.

^c Sum of unidentified fatty acids and/or those present in low concentrations.

^d Sum of polyunsaturated fatty acids.

^e Saturation index (sum of saturated acids/sum of unsaturated acids), not including epoxy 18:0.

Sources: Refs. 22 and 94.

lipids present in those tissues, but rat lung PE contained lower proportions of unsaturated fatty acids than *P. carinii* PE [94].

The other major *P. carinii* phospholipid class PC had similar 18:1 concentrations as PE, but it had six times more 16:0 (Table 6). Epoxy 18:0 was also detected in *P. carinii* PC, but at levels lower than those found in PE (K. Chin and E.S. Kaneshiro, unpublished).

The highly saturated fatty acid composition of SM and particularly high levels of 24:0 and 22:0 were distinct [28]. Rat lung SM had 27% 24:0 and 18% 22:0, which are consistent with the suggestion that most if not all SM is scavenged from the lung by *P. carinii*.

In addition to fatty acids, unsaturated fatty alcohols (glyceryl ethers) are present in *P. carinii* membrane bilayers [30], but their saturated counterparts dominate. The LCB of SM has a double bond and therefore contributes to bilayer fluidity. Cholesterol is known to enhance membrane fluidity. The distinct 24-alkysterols with branches extending from the sterol side chains deep within the bilayer are expected to have significant effects on the physical properties of *P. carinii* membrane bilayers.

VIII. Lipids Associated with Protein and Carbohydrate Macromolecules

A. GPI Anchors

The *Pneumocystis* major surface antigens and some other proteins apparently anchor into the membrane bilayer by inositol lipids. Inositol lipid anchors of *Pneumocystis* cell surface membrane components have yet to be identified and characterized by direct biochemical analysis, but support for inositol lipid anchors was provided by the attachment of recombinant *Pneumocystis* proteins to GPI anchors using heterologous systems [99,100] (see also Chap. 2).

B. Fatty Acids and Fatty Alcohols

The high-molecular-weight (HMW) major surface antigens are glycosylated [101] and lipidated [102]. The covalently linked lipids may enable these molecules to anchor into the membrane bilayer. *P. carinii* glycoproteins (gp) with molecular masses of 120, 110, 98, 80, and 56 kDa were purified by continuous-elution gel electrophoresis. With the exception of gp56, lipids were detected in these glycoproteins. The inability to detect lipids in gp56 indicated that the lipids found in the others were not due to contamination during the purification procedure. The sites and the amino acid residues within the protein where these lipids attach have yet to be identified; however, preliminary results indicate that major surface antigen isolated, as gp120 had the highest concentration of lipids. Amide-linked fatty acids in gp120 were 18:0>>16:0>18:1>22:0>20:0=24:0, which is not

like the profiles of *P. carinii* total lipid amide-linked fatty acid fraction or that of SM (Tables 5 and 6). A novel class of protein lipidation was detected; ester-linked saturated C₁₄, C₁₆, and C₁₈ fatty alcohols were identified in gp120 [102]. While 1-tetradecanol (C₁₄) was not detected in the alkyl glycerolipids, it was present in the free fatty alcohol fraction of *P. carinii* [30], which is probably the source in the cell for this novel lipid modification of *P. carinii* proteins.

IX. Host-Pathogen Interactions

Pneumocystis infection in corticosteroid-immunosuppressed rats was correlated with a reduction in BALF phospholipids and the phospholipid/protein ratio [8,103]. Reduction in total lipids, phospholipids, [104], disaturated PC [105], and sterols [106] were also reported in BALF from humans infected with *P. jirovecii*. Disaturated PC level in BALF from normal individuals was 2.5 µg/mL BALF, whereas it was only 0.5 µg/mL in BALF from HIV-positive patients with *Pneumocystis* pneumonia [105]. During 10 days of chemotherapy, it increased to 1.0 µg/mL BALF; adjuvant therapy with methylprednisolone did not have an effect on the concentration of this phospholipid in BALF [105]. Also, genetically immunodeficient *scid/scid* mice infected with *Pneumocystis* [107–109] and weanling rabbits spontaneously infected with *Pneumocystis* [108] had reduced BALF phospholipid/protein ratios. Total phospholipids and phospholipid/protein decreased in the large-aggregate surfactant fraction [109]. Total phospholipid content in the large-aggregate fraction from uninfected and infected mice were 311 and 165 µg per mouse, respectively (phospholipid/protein ratio was 3.57 and 2.09, respectively). The large-aggregate fraction accounted for 94% of total BALF phospholipids in uninfected mice, whereas that fraction accounted for only 63% of total BALF phospholipids in PcP mice. The phospholipid content in the small-aggregate fraction from uninfected and infected mice were 20 and 94 µg per mouse, respectively, and suggested that the infection enhanced the conversion of surfactant from large- to small-aggregate forms.

At least three potential reasons may explain the reduced lipid levels in BALF from PcP lungs. Phospholipase activity in BALF was found to increase from 6.29 to 15.8 nmol/min/mg protein in cortisone-treated uninfected and cortisone-treated *P. carinii*-infected rats, respectively [103]. Human BALF from HIV-positive patients with *Pneumocystis* pneumonia was also found to have higher phospholipase activity; the increase in enzyme activity was correlated with severity of infection [104]. Elevation of enzyme activity was apparently the direct effect of *P. jirovecii* infection, as the samples in that study were from HIV-immunocompromised patients (not corticosteroid-immunosuppressed). A decrease in PG with concomitant increase in *bis*(monoacylglycerol)/phosphate (Fig. 1) in rat BALF occurred upon *P. carinii* infection [110]. This observation

is consistent with increased phospholipase activity in the alveolus during infection. In PcP, one or more enzymes may be produced and/or activated; these may act on some but not all phospholipid substrates. Some other workers reported that the phospholipid level in BALF is not altered during infection [111,112]. The apparently conflicting results on BALF studies may be due to differences in protocols used for preparing the BALF material analyzed, different analytical methods used to quantify phospholipids, differential phospholipase secretion/activation under different conditions, or antibiotic treatment. It has been demonstrated that tetracycline increases the production of lung surfactant [113].

Increased phospholipase activity, however does not explain the reduction in human BALF of other lipid compounds such as sterols [106]. There were 16 and 3 μg sterols/mL BALF from normal individuals and patients with *Pneumocystis* pneumonia respectively—i.e., sterol concentration in PcP BALF was reduced by 80%. Dilution effects were ruled out by analyzing albumin and urea in equivalent samples. As similar total lipid levels were found in the lungs of immunosuppressed, uninfected rats and lungs with fulminant *P. carinii* infections [28], this suggested that within *P. carinii*-infected lungs, lipids were removed from the alveolar lining fluid by the organism. This is consistent with the high lipid concentration and apparent high rate of fatty acid catabolism in the organism [24,28]. Elevated levels of lysophosphatides and free fatty acids were not detected in BALF from *Pneumocystis*-infected lungs [104], but these are expected to be readily taken up by the organism as well [5,21] (K. Chin and E.S. Kaneshiro, unpublished).

Reduced lipid secretion by alveolar type II cells may also contribute to decreases in BALF lipids in PcP [114,115]. Exposure to *Pneumocystis* organisms and to isolated preparations of the organism's major surface antigen decreased phospholipid secretion by these mammalian lung cells in vitro.

In contrast to total lipids, it has been reported that total phospholipids, PC sterols, SM, and PG increased in BALF of lungs infected with *Pneumocystis* [8,116]. Increased SM synthesis or turnover by type II alveolar epithelial cells may account for some of the change. However, there may be other contributing factors. It is not known whether SM or PG can serve as substrates for the putative *P. carinii* phospholipase enzyme(s) in BALF, whether uptake rates of different complex lipids by the organism differ, and whether catabolic rates of sphingolipid amide-linked fatty acids and glycerolipid ester-linked fatty acids differ (for changes in lung surfactant, see also chapter 13).

X. Conclusions

Extensive data on *Pneumocystis* lipids have accumulated in the literature since the second edition of this book. We now know the identities of most lipids that are present and we have some idea of the lipid species that are scavenged from

the host and those synthesized de novo by *P. carinii* or remodeled by its metabolism. It is clear that the organism significantly alters the biochemical nature and function of the lung. It appears that *Pneumocystis* has the metabolic capacity to synthesize a lot of different lipid compounds, more than might have been imagined earlier. These baseline data now provide the information required for elucidating biosynthetic pathways and to investigate the properties of *P. carinii* enzymes involved in lipid metabolism. Information being generated in the *Pneumocystis* Genome Project allows investigation of specific *P. carinii* enzymes that catalyze lipid metabolism by recombinant protein technology. Work along these lines (such as expression in heterologous systems of enzymes representing potential drug targets) is just beginning.

Acknowledgments

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List of Abbreviations

BALF: bronchoalveolar lavage fluid
BMAGP: *bis*(monoacylglycero)phosphate
CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone
CL: cardiolipin, diphosphatidylglycerol
CoQ: Coenzyme Q, ubiquinone
DAPI: 4',6-diamidino-2-phenylindole
DCCD: *N,N'*dicyclohexylcarbodiimide
DG: diglycerides
DTT: dithiothreitol
EGTA: [ethylene-*bis*(oxyethylenenitrilo)]glycol tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
EST: expressed sequence tag
FFA: free fatty acids
GLC: gas-liquid chromatography
gp: glycoprotein
GPI: glycerophosphatidylinositol
HMG-CoA: 3-hydroxy-3-methylglutaryl Coenzyme A
HPLC: high-performance liquid chromatography
LCB: long chain base
LPC: lysophosphatidylcholine
MG: monoglycerides
MS: mass spectrometry
NBD: *N*-7-nitro-2,1,3-benzoxadiazol-4-yl

NMR: nuclear magnetic resonance
PA: phosphatidic acid
PC: phosphatidylcholine
PcP: *Pneumocystis* pneumonia
PE: phosphatidylethanolamine
PHBA: *p*-hydroxybenzoic acid
PG: phosphatidylglycerol
PI: phosphatidylinositol
PS: phosphatidylserine
PUFA: polyunsaturated fatty acid
SAM: *S*-adenosyl-L-methionine
SBI: sterol biosynthesis inhibitors
SE: steryl esters
SIV: simian immunodeficiency virus
SM: sphingomyelin
SMT: sterol C-24 methyl transferase
SP-A: surfactant protein A
S/U: saturated/unsaturated ratio
TLC: thin-layer chromatography
TG: triglycerides

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Polyamines, Iron, and *Pneumocystis carinii*

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I. Introduction

This chapter discusses experimental approaches to the development of improved therapies for *Pneumocystis* pneumonia (PcP) based on interference with *Pneumocystis* polyamine and iron metabolism. For both approaches there are drugs currently approved for other indications that exploit these approaches and are highly active in animal models of PcP.

For polyamines there is eflornithine (DL- α -difluoromethylornithine, or DFMO), which is approved for treating African sleeping sickness caused by *Trypanosoma brucei gambiense* and for suppression of facial hair growth in women. DFMO had been extensively examined clinically in the past as a therapy for PcP, but animal model data collected after those trials points to the possibility that modification of the mode of administration could enhance activity and reduce dosage. Beyond this compound and the particular polyamine target it exploits, other targets have been revealed by biochemical research and are discussed in this chapter. The effect *Pneumocystis* has on host plasma concentration of a polyamine precursor has promise as a new diagnostic tool.

Deferoxamine (DFO), an iron chelator approved for treating iron overload, shows strong activity in animal models of PcP. Pharmacokinetic data demonstrate that plasma concentrations of DFO effective in the rat model of PcP are far lower than the concentrations reached in humans treated for iron overload. Recent data

show that this chelator does not act by depriving *Pneumocystis* of the iron needed to grow, as had been presumed, but that DFO has a direct and irreversible effect on *Pneumocystis*. Because the effect is long-lasting, weekly treatment for 3 weeks is highly effective in the rat model. Although another class of chelator active in vitro is not active in vivo, the in vitro activity suggests that other compounds active in vivo will eventually be found.

II. Polyamines and *Pneumocystis*

A. Polyamines

These low-molecular-weight, positively charged compounds are ubiquitous in all living cells. The most important are putrescine, spermidine, and spermine (Fig. 1). Although much of the evidence is indirect, it is clear that polyamines play many important roles in cell growth and differentiation [1,2]. Inhibition of polyamine biosynthesis and other means of manipulating polyamine concentrations have been used as approaches for the development of chemotherapies to treat cancers and infectious diseases [3].

B. Clinical Experience Using dl - α -Difluoromethylornithine to Treat *Pneumocystis* Pneumonia

DFMO is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC), the first step in polyamine biosynthesis. This compound has been used in more preclinical and clinical investigations than any other antipolyamine agent. In 1983, six AIDS patients with PcP who had failed to respond to either trimethoprim-sulfamethoxazol (TMP-SMZ) or pentamidine were treated with DFMO; this treatment was successful for four of the six [4]. Subsequent open clinical trials using DFMO as a salvage treatment involved groups ranging in size from 31 to 345 patients. On an intent-to-treat basis, the overall clinical response rate in these open trials varied between 36 and 68% [5,6]. Controlled clinical trials reported a successful rate of completion of therapy with DFMO (39%), which was comparable to the control group treated with TMP-SMZ (40%). However, DFMO was most often discontinued due to a lack of response, whereas TMP-SMZ was most often discontinued due to toxic drug effects; i.e. 49% of patients failed to respond to DFMO, but only 21% failed to respond to TMP-SMZ [7]. Other than an abstract reporting the activity of a new antipolyamine compound in treating a mouse model of PcP [8], DFMO and bis-benzyl-spermidine, discussed below, are the only ones tested against PcP.

C. Polyamine Biosynthesis

The biosynthetic pathway in mammalian cells and many microorganisms is made up of four key enzymes (Fig. 1). Ornithine decarboxylase (ODC) forms putrescine

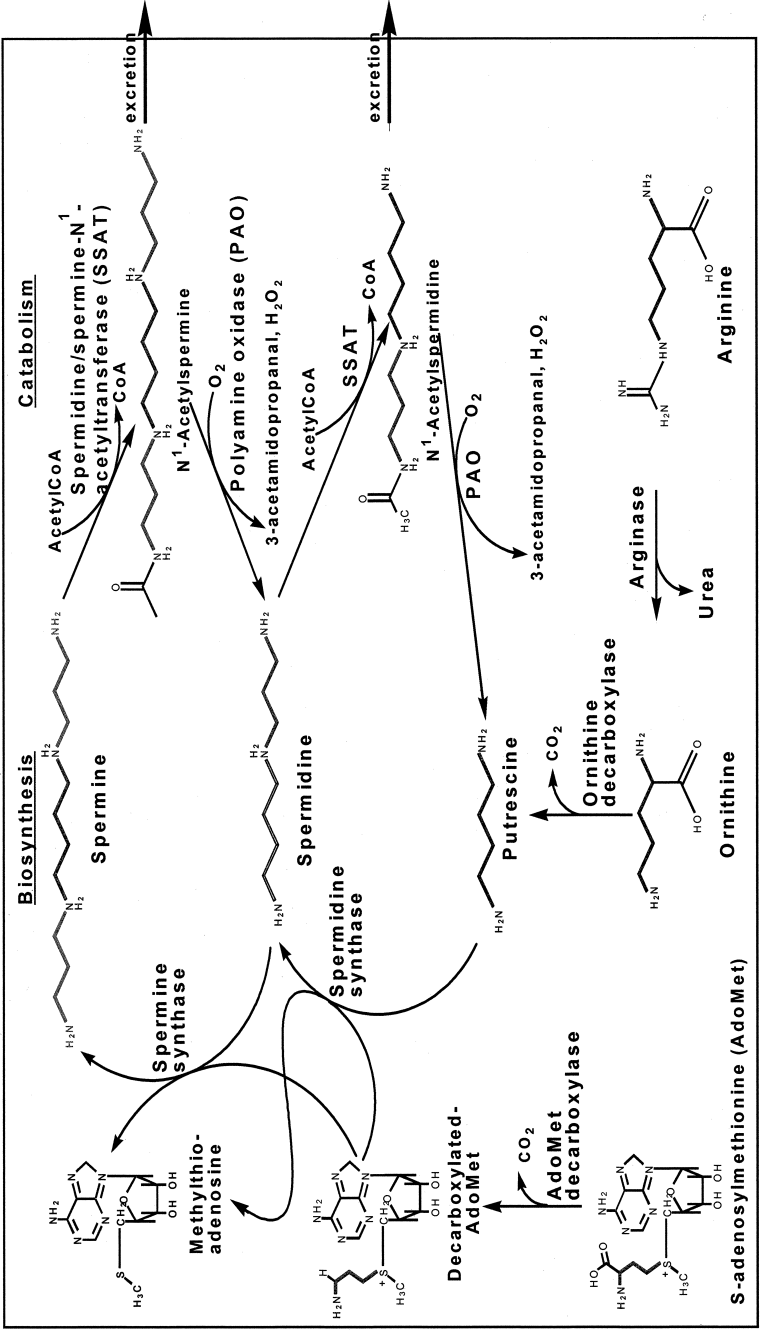


Figure 1 Polyamine metabolic pathway.

by decarboxylation of ornithine, a step that irreversibly commits this precursor to polyamine biosynthesis. *S*-adenosylmethionine decarboxylase (AdoMetDC) acts on *S*-adenosylmethionine (AdoMet) producing decarboxylated AdoMet (dcAdoMet), committing this compound to polyamine biosynthesis. dcAdoMet is the aminopropyl donor for the synthesis of spermidine from putrescine by spermidine synthase and for the synthesis of spermine from spermidine with the addition of a second aminopropyl group by spermine synthase. Both ODC and AdoMetDC are rate-limiting, highly inducible enzymes that respond strongly to polyamine elevation or depletion. The by-product of the aminopropyl transfer reactions is methylthioadenosine, which is cleaved by a specific phosphorylase, the products being recycled in various ways to methionine and purines, respectively.

D. Polyamine Interconversion and/or Loss

All of the four enzymatic steps above are highly exothermic and therefore essentially irreversible. However, many cell types can degradatively back-convert spermine into spermidine and spermidine into putrescine using two enzymes: spermidine/spermine- N^1 -acetyl transferase (SSAT) and polyamine oxidase (PAO) (Fig. 1). SSAT is cytosolic, generally highly inducible, and rate-limiting for this back-conversion. SSAT utilizes acetyl-CoA to acetylate terminal amino groups of spermidine or spermine, thus converting them into N^1 -acetyl derivatives. Acetylated polyamines are either excreted from the cell or oxidatively cleaved by PAO, a FAD-dependent enzyme that produces 3-acetoamidopropanal and spermidine or putrescine from N^1 -acetyl spermine or spermidine, respectively. The overall action of SSAT and PAO is to remove an aminopropyl group from spermine or spermidine.

Since acetylated polyamines are less charged at physiological pH than the parent compounds, they leave the cell more readily. Thus another effect of SSAT can be a net loss of polyamines. SSAT is critical for the maintenance of intracellular polyamine concentrations and is usually highly regulated. It has a rapid turnover rate ($t_{1/2} = 15$ min) in rats treated with thioacetamide to induce SSAT production [9]. SSAT can also be induced by a number of factors including environmental stress, exogenous polyamines, analogues of polyamines, growth hormones, and various toxic agents [10].

E. Manipulation of Polyamine Metabolism for Therapeutic Purposes

Depletion of polyamines has been shown to lead to inhibition of cell division, even to cell death if the depletion is severe [11]. Additionally, interference with polyamine metabolism can lead to perturbations in other pathways, such as protein or DNA methylation and purine/methionine metabolism [12]. Cell division is

associated with an increase in intracellular polyamine content, and all of the effects of polyamine depletion are more pronounced in rapidly dividing cells [13]; this explains the relevance of studies of polyamine metabolism to therapies for cancer and infectious disease. A truism is that studies of metabolism and the development of chemotherapeutic agents are synergistic, since metabolic studies provide targets for the design of therapeutic agents, and these agents provide tools for metabolic studies. For polyamine research, this synergism has been particularly strong. Interestingly, collaboration between academia and industry in the polyamine field has also been strong, with both producing important basic metabolic research, novel chemical compounds, and critical clinical research.

The single clearest achievement in antipolyamine therapy has been DFMO treatment of African trypanosomiasis caused by *Trypanosoma brucei gambiense*. This is the only disease for which the U.S. Food and Drug Administration (FDA) has approved DFMO as a treatment, and DFMO is the first new treatment for this disease in over 50 years. DFMO provides essentially a 100% cure rate, including late-stage disease involving severe central nervous system infection [14]. New antipolyamine compounds have shown promise of improved treatment with regard to lower dosage, which would lower costs, and to an expanded spectrum of activity to include treatment of *T. brucei rhodesiense* infections. The reasons for the selective action of polyamine biosynthesis inhibitors against trypanosomes are interesting. One is that ODC is not regulated in these cells and exists as a constitutive, noninducible protein; consequently, trypanosome ODC activity recovers from DFMO treatment only very slowly, whereas the inducible mammalian enzyme recovers rapidly [15]. Another reason is that the trypanosomes do not regulate the production of AdoMet, so that this metabolite builds up even when putrescine concentrations are low. High concentrations of AdoMet result in hypermethylation of proteins, and this interferes with enzyme regulation [16]. The selective action of DFMO against African trypanosomes is thus dependent on differences in polyamine biosynthesis control between the parasite and the host rather than differences in the biochemical pathways.

F. *Pneumocystis* Polyamine Metabolism

After the initial clinical success using DFMO to treat PcP, attempts were made to reproduce this response in an animal model so as to have a tool for developing improved antipolyamine therapies. Initially no activity was found [17]. With experience gained from research with DFMO and trypanosomes, Clarkson et al. [18] were able to demonstrate DFMO activity in a rat model of PcP. Initially, ODC, the enzyme target of DFMO, was reported absent in *Pneumocystis* [19], but evidence of polyamine biosynthesis was found [20]. Subsequently, the enzyme was detected and measured by using the classical radiolabel release assay after

removal of an interfering substance [21], with confirmation via a more sensitive and definitive HPLC assay [22].

Recent work on the polyamine content of *Pneumocystis* and the response to DFMO has clarified at least one of the reasons for the sensitivity of *Pneumocystis* to DFMO. When *Pneumocystis* isolated from infected rat lungs are exposed to DFMO in vitro, intracellular polyamine concentrations decline much more rapidly than in other cell types. A 3-hr exposure of *Pneumocystis* to DFMO in vitro reduced the putrescine, spermidine, and spermine concentrations by 88, 71, and 84%, respectively [22]. In contrast, 3 to 4 days were required to deplete mammalian cells of putrescine and spermidine; even then, spermine concentration was either not reduced or reduced by only 50%, depending on the cell types [23–26]. Since de novo polyamine synthesis in both *Pneumocystis* and mammalian cells is blocked by DFMO, these data led to the hypothesis that polyamine catabolism is not regulated in *Pneumocystis*, which would cause this pathogen to be particularly vulnerable to compounds that interfere with polyamine synthesis. [27].

To confirm this hypothesis, we showed that *Pneumocystis* has both of the conventional polyamine catalytic enzymes, SSAT and PAO. The specific activity of SSAT in *Pneumocystis*, $1.78 \pm 0.5 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$, is similar to that reported in mammalian cells, $1.27 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$. However, the K_m for spermidine, $25 \text{ }\mu\text{M}$, is significantly lower than that for mammalian cells, $130 \text{ }\mu\text{M}$ [28]. The specific activity of PAO in *Pneumocystis*, $6.42 \pm 0.8 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$, is lower than that reported in mammalian cells, $34 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$. The K_m for N¹-acetylspermidine, 14.25 nM , is significantly lower than that reported for mammalian cells, $14 \text{ }\mu\text{M}$ [29].

The unusual feature of *Pneumocystis* polyamine catabolism is the lack of modulation of SSAT and PAO activities when the cellular content of polyamines is sharply reduced by exposure to DFMO. In mammalian cells, the effect of DFMO on polyamine content is attenuated by downregulation of SSAT, which slows polyamine catabolism, thus helping the cells to maintain the polyamines already synthesized. For example, exposure of mammalian CHO cells to 1 mM DFMO resulted in a 60% reduction in SSAT activity by 24 hr; putrescine and spermidine were substantially depleted after 3 days, but even then spermine was only slightly reduced [30]. This contrasts sharply to *Pneumocystis*, where a 3-hr exposure to 1 mM DFMO caused only minimally reduced SSAT and PAO activities (11 and 4%, respectively) while causing severe reductions in the content of all three polyamines.

Even though *Pneumocystis* does not repress SSAT when polyamines decline, the possibility of induction was examined, since a drug-induced increase in SSAT activity could enhance the therapeutic activity of compounds that block ODC. However, N¹, N¹²-diethylspermine, which is known to induce SSAT in L1210 cells [31], was without effect in *Pneumocystis*. These results suggest that

Pneumocystis SSAT is a nonregulated, constitutively expressed enzyme—a situation that has not been reported for any other cell type.

An expected consequence of polyamine acetylation is loss of acetylated polyamines from the cell due to the reduction in positive charge caused by the acetylation. Excretion of acetylated polyamines by *Pneumocystis* was examined by measuring the accumulation of acetylated polyamines in culture medium. On a basis of $\text{pmol/mg protein}^{-1}$, the total acetylated polyamine accumulation in the medium over 48 hr was 20% of the total normal polyamine content of the *Pneumocystis* cells. This excretion is similar to that reported in HT115 cells; 25% of the polyamine content was lost after 96 hr [32]. The addition of 1 mM DFMO for 12 hr reduced excretion by only 29% compared to untreated controls; this reduction was most likely due to the declining gradient across the cell membrane caused by the inability to make new polyamines as acetylated polyamines were lost.

G. Continuous DFMO Infusion and Increased Efficacy Against *Pneumocystis* Pneumonia

As mentioned above, in *Pneumocystis* DFMO causes all three polyamines to become severely depleted within 3 hr—a much faster drop than observed in any other cell type. Furthermore, upon removal of DFMO, polyamine concentrations approach pretreatment concentrations within 1 hr [22]. The combination of this observation with knowledge of the short 1-hr plasma half-life in rats [33] led to the predictions that administration of a single daily bolus would be the least effective means of treatment, administration in the drinking water would be more effective, and continuous infusion of DFMO would be most effective. These predictions were confirmed. Daily bolus administration of DFMO up to $3000 \text{ mg kg}^{-1} \text{ day}^{-1}$ had no effect on PcP in the animal model. An absorbed dose of $1,267 \text{ mg kg}^{-1} \text{ day}^{-1}$ from administration of a 1% solution in the drinking water was associated with a reduction in cyst count, but this reduction was not statistically significant. In contrast, a small dose administered by implanted pumps ($418 \text{ mg kg}^{-1} \text{ day}^{-1}$) produced a strong, statistically significant response as judged by (1) the count of *Pneumocystis* cysts remaining in the lungs; (2) the ratio of lung weight to control, uninfected lung weight; and (3) the number of animals surviving for the full treatment period. The response to continuous infusion, 91% cyst suppression, was similar to the previously observed responses to 2, 3, and 4% solutions of DFMO in the drinking water—i.e., 88, 97, and 96% cyst suppression, respectively [34]. Since these oral doses calculate to absorbed doses of 2820, 3693, and $4341 \text{ mg kg}^{-1} \text{ day}^{-1}$, respectively, the response to continuous infusion is approximately seven- to ninefold better than administration in the drinking water. Although a 91% reduction in lung cyst burden is not a cure of PcP in the rat model, clinical cures are obtained in patients treated with

this drug, as discussed above. Considering that the plasma half-life of elimination in humans is three times longer than in rats [33,35,36], the DFMO plasma concentration achieved in patients given the standard dosage of $400 \text{ mg kg}^{-1} \text{ day}^{-1}$ is likely to be considerably higher than that achieved in rats given $418 \text{ mg kg}^{-1} \text{ day}^{-1}$.

The standard clinical investigational protocol for DFMO treatment involves intravenous administration of 100 mg kg^{-1} over at least 20 min repeated at 6-hr intervals for 10 to 14 days, followed by 75 mg kg^{-1} every 6 hr for 4 to 6 weeks [37]. The data presented above suggest that a better response might be achieved if the drug were administered by continuous infusion. Reviews and primary papers covering the use of DFMO as a salvage therapy for patients with *Pneumocystis* pneumonia failing other therapies included the following numbers of patients: 345 [38], 42 [39], 31 [40], and 31 [5]. On an intent-to-treat basis, the overall response rates were 36, 55, 68, and 45%, respectively. All these studies used the four-times-a-day treatment schedule except the study that produced the 68% response rate [40], which used continuous infusion. In the only clinical study using DFMO as initial therapy [7], also the only controlled study with patients randomly assigned to the DFMO treatment group (51 patients) or to a control group receiving standard TMP-SMZ therapy (47 patients), administration was by continuous infusion and the response rate observed was 39%, equal to the response to TMP-SMZ in that study (40%).

The ability of *Pneumocystis* to rapidly restore polyamine concentrations after exposure to DFMO [22] suggests that the limitation of effectiveness relates to the trough drug concentration rather than either the peak or the mean. Administration of 100 mg kg^{-1} intravenously every 6 hr to patients treated for PcP produced peak plasma concentrations ranging from 196.6 to $317.9 \mu\text{g mL}^{-1}$ and troughs ranging from 71.3 to $113.3 \mu\text{g mL}^{-1}$ [41].

Administration of 200 mg kg^{-1} every 12 hr, the same total daily dose, to adult patients with sleeping sickness produced trough plasma concentrations ranging from 4.2 to $46.4 \mu\text{g mL}^{-1}$ [42]. Therefore the cause for some of the variability in the response of PcP to DFMO could reside in variable drug troughs. It is possible that continuous infusion at rates appropriate for individual patients would produce either an enhanced response or fewer adverse effects or possibly both. However, this is a question that cannot be answered with existing data. Another issue regarding the clinical utility of DFMO is tolerance and toxicity.

H. Bis-benzyl Polyamine Analogue

As already mentioned, since *Pneumocystis* polyamine catabolism is not regulated, this fungus becomes vulnerable to inhibition of the polyamine biosynthesis pathway. There are inhibitors of other polyamine biosynthetic steps as well as compounds that cause acceleration of polyamine degradation or interfere with poly-

mine function [43]. Among these is a series of bis-benzyl polyamine analogues synthesized by Merrell Dow Pharmaceuticals, now incorporated into Hoechst, Marion, Roussel. We examined one of these bis-benzyl derivatives [MDL 27695, which is (*N,N'*-bis{3-[(phenylmethyl)amino]propyl}-1,7-diaminoheptane or $\text{C}_6\text{H}_5\text{CH}_2\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_7\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{C}_6\text{H}_5$, or BBS)] for the ability to modulate the polyamine concentrations of *Pneumocystis*, to induce the polyamine catabolic enzyme spermine-spermidine acetyl transferase (SSAT), to block growth in vitro, and to treat an animal model of PcP. BBS did not induce SSAT activity and caused only a minor reduction in the polyamine content of rat-derived *P. carinii*. However, transport of BBS could be blocked by spermidine in vitro, and a 3-week treatment of a rat model with a dose of $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ reduced the lung cyst count by 97%. These data, plus the total inability of *Pneumocystis* to metabolize BBS, suggested that this compound interferes with polyamine function in *Pneumocystis* [44]. Such a mode of action indicates the presence of a novel chemotherapeutic target.

I. Inhibition of AdoMetDC

Although DFMO is widely used for modulating polyamine metabolism in vitro and in vivo, it has limitations. In mammalian cells, DFMO is able to cause a major reduction in putrescine and spermidine pools, but spermine declines slowly or not at all. Reduction in putrescine and spermidine results in inhibition of cell proliferation within two cycles, but the continuing presence of spermine allows the cells to survive [45]. This persistence of spermine is attributed to a combination of an incomplete inhibition of ODC [46]; downregulation of SSAT, preventing polyamine catabolism [47]; and the continuing presence of dcAdoMet, which drives the conversion of polyamines toward spermine [48]. Thus the effect of DFMO on mammalian cell growth is cytostatic rather than cytotoxic. Inhibitors of other enzymes in the polyamine pathway have been synthesized with the aim of achieving more complete polyamine depletion in target cells.

AdoMetDC is also a rate-limiting enzyme in polyamine biosynthesis and has been a target for drug development. The earliest inhibitors of AdoMetDC caused the expected decline of spermidine and spermine pools, but there was a vast increase in putrescine due to ODC being derepressed by the decrease in spermidine and spermine. Thus putrescine can rise more than the decline of higher polyamines, so that the total polyamines are actually increased [49]. Despite this, some AdoMetDC inhibitors do have antitumor and antiparasitic activity. One class of AdoMetDC inhibitors comprises derivatives of one of the earliest compounds known to inhibit this enzyme, methylglyoxal bis(guanyldrazone) (MGBG). MGBG is an effective inhibitor, but it has problems. One is toxicity associated with damage to mammalian mitochondria. Another is that MGBG inhibits serum diamine oxidase, an enzyme that mediates extracellular polyamine

catabolism. Inhibition of diamine oxidase reduces the natural rate of extracellular polyamine degradation countering biosynthesis inhibition. In a search for good inhibitors of AdoMetDC that do not damage mitochondria, Regenass et al. used a traditional structure-activity relationship and produced several promising compounds [50,51]. One of these, CGP-48664, is a powerful inhibitor of AdoMetDC; it has an I_{50} of 5 nM and inhibits diamine oxidase less than MGBG. CGP-48664 caused a major depletion of spermidine and spermine in mouse L1210 leukemia cells, but as for MGBG, the putrescine pools increased. Despite this, CGP-48664 has improved antitumor activity and a better chemotherapeutic index [50,51]. Another in this series, CGP-40215, has excellent activity against African trypanosomes and low host-cell toxicity in vitro [52,53]. CGP-40215 is also effective in a mouse model of PcP. Treatment with 50 mg kg⁻¹ once a week was as effective in reducing cyst burden as TMP-SMZ at 50 and 250 mg kg⁻¹ administered 5 days a week [8]. Thus AdoMetDC is a viable target for drugs to treat PcP.

J. S-Adenosylmethionine

As discussed above, S-adenosylmethionine (AdoMet or SAM or SAMe) is not only key to polyamine biosynthesis but also plays a larger role as methyl donor in a myriad of biochemical processes. Reactions requiring AdoMet include methyl-group transfers for the formation of phosphatidylcholine, regeneration of methionine, methylation of phospholipids, methylation of other small molecules, and methylation of macromolecules including proteins, DNA, and RNA [54]. It is estimated that in mammalian cells, the flux of AdoMet is 99% toward transmethylation reactions [55] and only 1% toward polyamine formation [56]. Manipulation of this key metabolite has been an approach to the treatment of cancer [46] and parasitic diseases such as trypanosomiasis [57]. Little is known about the fate of AdoMet in *Pneumocystis*. However, the rapid synthesis of polyamines by this fungus [58] suggests that the metabolic partition could be very different than in mammalian cells.

Figure 2 outlines most of the reactions associated with AdoMet. Synthesis is catalyzed by the enzyme AdoMet synthetase, which transfers the adenosyl group of ATP to methionine. The residual triphosphate group is hydrolyzed into P_i and PP_i by the intrinsic polyphosphatase activity of AdoMet synthetase. When AdoMet is used as a methyl donor, it is converted into S-adenosylhomocysteine (AdoHcy). AdoHcy is then hydrolyzed by AdoHcy hydrolase to form adenosine and homocysteine (Hcy). Adenosine can either be deaminated by adenosine deaminase to inosine or be phosphorylated by adenosine kinase to form AMP. Hcy is either conjugated with serine by cystathionine synthase to form cystathionine, which is further metabolized to cysteine via the transsulfuration pathway (not shown in Fig. 2), or is remethylated by 5-methyltetrahydrofolate to methionine, which can be recycled back to AdoMet. (Thus the folate pathway can be used

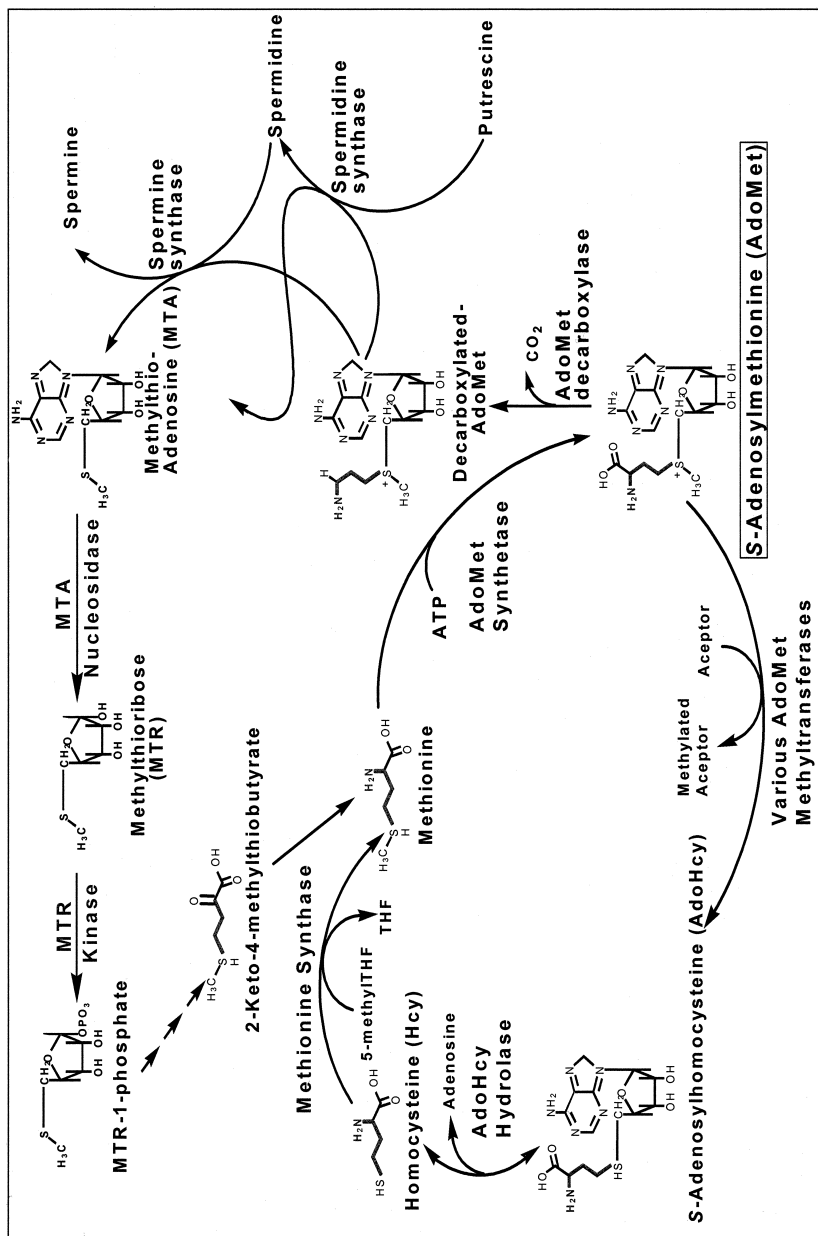


Figure 2 S-adenosylmethionine metabolic pathway.

to replenish AdoMet.) For polyamine biosynthesis, AdoMet is decarboxylated by AdoMet decarboxylase (AdoMetDC), producing decarboxylated AdoMet (dcAdoMet), which is committed to polyamine biosynthesis. dcAdoMet donates the aminopropyl group needed to form spermidine from putrescine and spermine from spermidine. The end product of the aminopropyl transfer reactions is methylthioadenosine, which is cleaved by a specific phosphorylase, the products being recycled in various ways to methionine and purines, respectively.

K. S-Adenosylmethionine and *Pneumocystis*

Except for mutants, to our knowledge no cell has previously been reported to lack AdoMet synthetase, and AdoMet has not been known to be an essential nutrient for any cell. It is, however, an essential metabolite. A mutation of *Escherichia coli* that results in very low AdoMet synthetase activity causes a cell division defect [59]. Knockout of *Saccharomyces cerevisiae* AdoMet synthetase is lethal, but the knockout can be rescued by exogenous AdoMet [60]. Despite the universal activity of cytoplasmic AdoMet synthetase, exogenous AdoMet can have an effect on cells. For example, for *S. cerevisiae*, added AdoMet can stimulate budding and outgrowth as well as RNA and protein synthesis [61]. Liver cirrhosis causes low AdoMet synthetase activity, and AdoMet diet supplementation has been shown to enhance patient recovery [62,63].

Since AdoMet had been reported to enhance the growth of another fungus [61], we added AdoMet to the medium we used for *Pneumocystis* culture (see Sec. III, on iron, for an additional discussion of the axenic culture). This caused a qualitative and quantitative improvement in *Pneumocystis* culture, including the production of cysts. Because of the effect of AdoMet on *Pneumocystis* culture and because analogues of AdoMet have been explored as pharmaceutical leads, we chose to examine the AdoMet metabolism of *Pneumocystis* in more detail. We found that *Pneumocystis* is an AdoMet auxotroph, a condition not previously reported for any cell [64]. This conclusion is supported by the following. No AdoMet synthetase activity could be detected in cell extracts using a sensitive radioisotope incorporation assay; this easily detected the enzyme in host cell extracts [64]. AdoMet is required for axenic growth in that replacement of horse serum in the medium with serum depleted of AdoMet (dialyzed horse serum) or low in AdoMet (fetal bovine serum) resulted in a 98% decline in cell number as compared to cells supplied with AdoMet. *Pneumocystis* has a high-affinity, highly specific transporter for AdoMet. We measured a K_m of 4.5 μ M and pH optimum of 7.5. The related compounds L-methionine, ATP, hypoxanthine, adenine, S-adenosylhomocysteine, methylthioadenosine, S-adenosylethionine, and decarboxylated AdoMet were not competitive with AdoMet uptake, indicating that the transporter is specific for AdoMet. Sodium azide (20 mM) inhibited AdoMet uptake by $24 \pm 4\%$, and the combination of 20 mM sodium azide and 1 mM

salicylhydroxamic acid (SHAM) inhibited uptake by $95 \pm 3\%$; these results indicate that transport is active and the required energy is dependent on both a cytochrome chain and an alternative oxidase [64]. Based on the measured decline of AdoMet in medium incubated with cells for 8 hr, we calculated that AdoMet is used at the rate of 1.40×10^7 molecules cell⁻¹ min⁻¹.

An excellent chemotherapeutic opportunity is presented by this dependency on AdoMet and the presence of a specific transporter. Although normal metabolites related to AdoMet do not inhibit transport, it may be possible to design analogues that are recognized by the transporter with equal or greater affinity than that of AdoMet. If transported in place of AdoMet, such compounds could also be expected to interfere with AdoMet-dependent methylation and polyamine biosynthesis. This dual mode of action would be synergistic, because suppression of AdoMet transport will increase the ratio of analogue to AdoMet within *Pneumocystis*, thus enhancing the effect of the analogue on AdoMet-utilizing enzymes. Because mammalian cells do not actively transport AdoMet, the chances of developing a compound with high specificity for *Pneumocystis* would seem to be good. Analogues of AdoMet have already been synthesized and considered as pharmaceutical leads for other infectious agents [57]. We tested two such analogues for their ability to inhibit AdoMet transport. The synthetic compound S-adenosylethionine (the S-methyl group of AdoMet is substituted by an S-ethyl group) does not compete with AdoMet for transport. But in a preliminary experiment, sinefungin (a natural nucleoside antibiotic with the sulfonium group of AdoMet replaced by an amino-substituted methylene group) at 100 μ M inhibited AdoMet transport by 98%. This is a promising result in that it demonstrates that AdoMet transport can be blocked and it serves as a lead for further development of inhibitors.

L. S-Adenosylmethionine Depletion in the Host

Based on the observation that AdoMet is required in and is depleted from culture medium, we considered the possibility that *Pneumocystis* might cause a reduction in the plasma AdoMet concentration of infected animals. Concentrations of most plasma components are held relatively constant by a negative feedback system that would limit changes induced by a pathogen. However, since all individual mammalian cells synthesize AdoMet, we thought it possible that AdoMet could be an exception, so that an increase in utilization from the plasma would be reflected by a decrease in the plasma concentration. To test this, we compared the results of plasma AdoMet analysis to counts of *Pneumocystis* in lung, and a striking inverse and essentially linear correlation was found [64]; the animals with highest number of parasites in the lungs had undetectable plasma AdoMet (<1 nM). We showed that these changes in plasma AdoMet were not caused by immunosuppression or other aspects

of the model. Plasma AdoMet of three animals that were chronically immunosuppressed but not infected and three animals that had had PcP but were treated with TMP-SMZ were $35 \pm 6 \mu\text{M}$ and $27 \pm 5 \mu\text{M}$, respectively—concentrations similar those of six healthy rats ($29 \pm 7 \mu\text{M}$). The effect on plasma AdoMet is so striking that we used the AdoMet consumption rate measured in vitro [64] to calculate how quickly we could expect *Pneumocystis* to deplete AdoMet in the extracellular fluid of a rat. Based on the measured consumption rate of AdoMet per *Pneumocystis* cell in vitro, the peak number of *Pneumocystis* cells per rat and the rough approximation that 20% of the weight of a rat is extracellular fluid, we calculate that in 36 min *Pneumocystis* can consume an amount of AdoMet equal to the total AdoMet in the extracellular fluid of a rat.

M. Plasma S-Adenosylmethionine Concentrations, a Potential Aid for the Rapid Diagnosis of *Pneumocystis* Pneumonia and Measurement of Response to Therapy

Plasma AdoMet becomes depleted in humans with PcP, just as in rats [65]. Of 15 patients with probable or confirmed PcP, 14 had plasma AdoMet concentrations below the limit of detection. The remaining patient with PcP had detectable AdoMet in the plasma but a severely reduced concentration compared to all other subjects without PcP. No other group of patients who were HIV positive (bacterial pneumonia, asymptomatic, *Cryptococcus* infection, tuberculosis) showed a significant reduction in plasma AdoMet. The consistency of pattern of association and the return of plasma AdoMet to normal values with effective treatment suggest that severe depletion of AdoMet is characteristic of PcP. As a potential diagnostic test in the population studied, reduced plasma AdoMet was sensitive and specific, possessing both high positive and negative predictive value. A commentary on this work published at the same time [66] noted that current cytological methods of PcP diagnosis often detect tuberculosis, bacterial pneumonia and lung cancer, diseases common in patients suspected of having PcP. If further clinical studies confirm that measurement of plasma AdoMet provides a reliable means of diagnosing PcP, it would not detect these other conditions. However, if *Pneumocystis* is found in a sample from a patient with pneumonia and the disease etiology is assumed to be *Pneumocystis*, the correct etiology of the pneumonia may be missed if the patient is colonized by this fungus only. Depletion of plasma AdoMet would indicate pneumocystosis and not colonization.

III. Iron and *Pneumocystis*

A. Iron and Biology

Iron is an essential element for all known cells with the classical exception of *Lactobacillus* and the more recent finding for *Borrelia burgdorferi* [67]. The

roles of iron include functioning as a ligand-binding moiety and as a redox element. Because the redox shift is easily achieved under physiological conditions, iron is an important enzyme cofactor. However, this very property also causes iron to be toxic due to the ease with which it generates reactive oxygen species. (For reviews, see Refs. 68–73.)

An interesting aspect of iron is the relatively poor bioavailability of this highly abundant element. For microbes in soil, even soil in which iron is plentiful, extraordinary effort is required to obtain iron. Many microbes synthesize and release siderophores, small molecules that complex strongly with environmental iron, making it soluble. Siderophore-secreting cells have transporters that recognize the siderophore-iron complex, allowing the cells to retrieve iron from their environment. (For reviews, see Refs. 74 and 75.) Mammals also have specialized mechanisms for obtaining iron. Through expression of a receptor on the surface of enterocytes, needed iron is taken up from the gut; when iron requirements have been met, expression of this receptor is downregulated to prevent iron overload. From enterocytes, iron is transferred to plasma transferrin, allowing distribution to other parts of the body as an iron-transferrin complex. When individual cells need iron, they express receptors for the transferrin/iron complex. A pH reduction in endocytic vesicles causes the iron/transferrin complex to dissociate and transferrin to be recycled out of the cell, with the released iron remaining in the cytoplasm. Within the cytoplasm, iron is bound tightly to other proteins to avoid uncontrolled generation of reactive oxygen species. (For reviews on iron acquisition and transport by mammals, see Refs. 71 and 72.)

B. Iron Storage in Mammals

Should excess iron be present in mammals, there are mechanisms for sequestration and storage of the excess, although these mechanisms have limited capacity. One is transferrin itself, which is generally in adequate abundance to remain unsaturated, so that excess plasma iron can be quickly adsorbed. Should transferrin begin to become saturated, production is upregulated and, in addition, iron is transferred to ferritin, a storage protein. Lactoferrin is another mammalian iron-binding plasma protein, and its production is upregulated as a nonspecific response to infection. Lactoferrin binds iron more tightly than transferrin, so that an increase in plasma lactoferrin results in a reduction of iron available to infecting organisms. Iron-loaded lactoferrin is removed from plasma by Kupffer cells, with the excess eventually moved to ferritin within hepatocytes, where it is completely unavailable. (For a review of iron storage in mammals, see Refs. 71 and 72.)

In contrast to their highly effective means of iron sequestration, mammals have no ability to remove excess iron from the body other than by blood loss and a small amount through normal loss of other cell types. Should a mammal become overloaded with iron to the extent that the sequestration mechanisms

become saturated, iron is toxic. Overload can occur from excessive iron in the diet, excessive iron dietary supplementation, a genetic condition that causes aberrant iron absorption from a normal diet (essential hemochromatosis), or repeated transfusions, such as those required for the treatment of β -thalassemia (a genetic condition that prevents production of mature hemoglobin β chains and thus functional erythrocytes). For some conditions, phlebotomy is an effective treatment, but obviously neither for β -thalassemia nor for accidental iron poisoning from dietary supplements. About 40 years ago, the iron chelator deferoxamine (DFO, or desferrioxamine) was approved for treating iron overload. DFO is a modified bacterial siderophore, which, when administered to iron-overloaded patients, complexes with iron and is excreted in the urine and bile as the DFO-iron complex feroxamine. DFO remains the only drug approved for treatment of iron overload. Patients with β -thalassemia are treated daily with DFO infusion, beginning in early childhood, and this treatment must be continued throughout their lives. With strict compliance, these patients can approach a normal lifespan. (For reviews on iron overload and treatment, see Refs. 76 to 78.)

C. Initial Observations on DFO and *Pneumocystis* Pneumonia

Considering that iron is an essential element for nearly all cells and that mammals respond to infection by sharply decreasing the supply of iron available to infecting microbes, the idea of augmenting this response by causing an even stricter reduction in iron availability is reasonable and was proposed long ago. (For reviews, see Refs. 79 to 82.) DFO was tested against malaria and was found to be active against the most virulent of human malarias in vitro, that caused by *Plasmodium falciparum* [83]. DFO was subsequently shown to be clinically active as well [84–86]. We tested DFO against *Pneumocystis* in the rat model of PcP and found it active against this fungus [87]. Subsequently but independently, Weinberg and Shaw [88] also found DFO active against *Pneumocystis* in culture; 15 μg DFO mL^{-1} (23 μM) was as effective in suppressing *Pneumocystis* growth as 50 μg trimethoprim (TMP) mL^{-1} in combination with 250 μg sulfamethoxazole (SMZ) mL^{-1} . Weinberg later tested DFO in vivo, finding good activity in the mouse model of PcP but weak activity in the rat model, even at doses greater than those providing strong activity in the mouse model [89]. We had used a yet higher dose in the rat model and observed a strong response [87]. In that same paper, Weinberg reported more extensive culture experiments that produced overall results similar to his earlier brief report. He also included a series of hydroxypyridone iron chelators, unrelated to DFO, and found them active against *Pneumocystis* in vitro but not in vivo. Boelaert et al. [90] tested the lead hydroxypyridone (L1 or CP20) and one other and found both inactive in vivo. We also found L1 ineffective in vivo (unpublished).

Weinberg reasoned that if these compounds were acting as chelators, loading them with iron should abolish activity, and it did for both DFO and the hydroxypyridones in vitro. Boelaert et al. also reported that the DFO-iron complex was also inactive in vivo [91]. However, it must be noted that the iron-chelator complex differs from the free chelator in more ways than in the ability to chelate additional iron. The molecular confirmation of DFO-iron is markedly different from that of free DFO. The chelating sites of both DFO and the hydroxypyridones can undergo redox reactions that could be the basis of their anti-*Pneumocystis* activity; such reactivities would be inhibited by binding to iron. However, the structure of DFO and the hydroxypyridones are markedly different and that the fact that both are active when uncomplexed to iron and inactive when bound to iron does support iron chelation as the mode of action. Other observations supported the role of iron availability and development of PcP. The mammalian plasma protein lactoferrin has direct anti-*Pneumocystis* activity in vitro when used either as a single agent or in combination with compounds with known anti-PcP activity [92]. Non-transferrin-bound iron is increased in patients with PcP [93]. As HIV disease progresses, lactoferrin plasma concentrations decline, and this decline has been suggested to add to *Pneumocystis* susceptibility beyond the immunosuppression caused by loss of CD4 cells [94]. All of the above observations support the hypothesis that DFO acts against *Pneumocystis* by reducing available nutritional iron.

D. DFO Infusion and *Pneumocystis* Pneumonia

If DFO acted against *Pneumocystis* by deprivation of iron, the method of daily bolus intraperitoneal injection that we used for administration would not be optimal. Rather, a dosage schedule and route that would ensure that DFO is present at all times, at a concentration just sufficient to virtually eliminate free iron, should cause the maximum *Pneumocystis* iron deprivation and have minimal host toxicity. The short half-life of DFO [95], particularly in rats [96], exacerbates the effect of bolus administration. Constant infusion was therefore predicted to markedly increase the efficacy of DFO. To test this, infusion pumps were implanted in rats and PcP was induced by immunosuppression and intratracheal inoculation. Infusion was at mean rates of either 195 or 359 mg kg⁻¹ day⁻¹ for 3 weeks. Unexpectedly, there was no significant improvement in the dose response [97].

E. Clinically Achievable Plasma DFO Effective in the Animal Model of *Pneumocystis* Pneumonia

Some very positive information was gained using these pumps, however. The design of the pumps did assure that, although the rates varied from pump to pump, the rate for a given pump was constant. Consequently, when we measured

the plasma DFO at the time of sacrifice, these final plasma concentrations were representative of the plasma DFO content over the experimental period. For animals that had plasma DFO $\geq 1.5 \mu\text{g mL}^{-1}$ at the end of the treatment period, we found no trophozoites remaining in the lungs. We could detect low numbers of cysts in some animals, but the proliferative trophozoite form was undetectable. This is of particular significance because the 500 to $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$ dosage we used to treat the rat model cannot be tolerated by humans, but the well-tolerated human dose of 50 mg kg^{-1} infused day^{-1} produces plasma concentrations of $>4.0 \mu\text{g mL}^{-1}$ [98,99].

F. Development of Axenic Culture System

Because continuous infusion did not produce the effect predicted by the hypothesis that DFO works by depriving *Pneumocystis* of nutritional iron, a further test of this hypothesis was made. This involved short-term exposure of *Pneumocystis* to DFO in culture. The reasoning was that an exposure to low iron conditions should not have a serious adverse effect on *Pneumocystis* provided that nutritional iron was restored after a brief time. However, the then standard method of short-term culture of *Pneumocystis* with a layer of mammalian cells was not suitable for testing the effect of short-term iron deprivation. One reason was the intimate association of *Pneumocystis* with host cells, since such an association could allow *Pneumocystis* to obtain iron from the host cells even if iron were unavailable in the culture medium due to DFO. Another was that, in a system requiring the presence of feeder cells, it is not possible to separate the effect of DFO directly on *Pneumocystis* from an indirect effect via the feeder cells. For these reasons we attempted to develop a system that did not require a feeder cell—an axenic system or monoculture.

Potential functions of the feeder layers of *Pneumocystis* cultures include nutrition as well as a stratum for attachment. One approach to providing adequate nutrition is a rich medium with a high concentration of serum that is changed frequently to avoid depletion of any critical nutrient that may be present at a low concentration. However, frequent changes of medium also disturb the cultured *Pneumocystis*, and some *Pneumocystis* cells would likely be removed with spent medium, thus obscuring detection of growth. To circumvent this, we used tissue culture well inserts with porous bottoms so that the medium could be changed below the insert without disturbing the *Pneumocystis* held in the insert. To provide an attachment substrate, we used inserts with collagen-coated membranes. We also experimented with the culture medium by adding such compounds as *p*-aminobenzoic acid, N-acetylglucosamine, ferric pyrophosphate, as well as extra cysteine and glutamine. Because we had found that polyamine synthesis is very active in *Pneumocystis*, as discussed earlier in this review, we also added the polyamine precursors putrescine and S-adenosylmethionine (AdoMet). Of all

these extra nutrients, AdoMet was clearly the most helpful in producing cysts, supporting growth, and maintaining the microscopic appearance of cultured cells. The role of AdoMet in *Pneumocystis* is discussed more fully in Section II.A of this chapter.

Our initial intention was simply to develop an axenic system for short-term culture, yet the cells seemed to continue to grow far beyond anything reported previously. On several occasions, we had continuous growth over a 4-month period. These initial successes convinced us that we had achieved a true continuous culture system. Since the time of the publication reporting that conclusion [100], we have found that, although growth is better in some hands than in others, most workers are unable to maintain growth beyond a few weeks (personal communications) and a recent careful study showed no growth at all using the system we described [101]. Even in our hands, results have become variable and we have not been able to use the culture system as a replacement for *Pneumocystis* isolated from rat lungs.

Regardless of these limitations, which will be more fully described in a separate publication, this axenic system did allow us to test the effect of DFO on *Pneumocystis* free from interference by any cocultured cells. Results described below led to information on the mechanism of action and a prediction of in vivo activity that was confirmed. Other in vitro results have subsequently been confirmed by in vivo data. Our in vitro observation that polyamines of *Pneumocystis* rapidly become depleted upon exposure to DFMO but recover very quickly upon removal of the drug led to the important observation regarding efficacy and pharmacokinetics discussed earlier in this chapter. If mammalian cells had been present, the in vitro data would not have been clear. The requirement of AdoMet in vitro led to the observation that the plasma of rats infected with *Pneumocystis* becomes depleted of this critical metabolic intermediate [102], and this led to the clinical study discussed earlier. Because *Pneumocystis* is the only known AdoMet auxotroph, this added to our confidence that we had the key to continuous culture. Now we believe it will prove to be one of the keys but there are others yet to be found. Nevertheless, the axenic system we described should prove useful for in vitro investigations.

G. Short-Term Exposure of *Pneumocystis* to DFO in Vitro

Based on the hypothesis that DFO deprived *Pneumocystis* of the iron it required for growth, exposure of cultured *Pneumocystis* to DFO for the relatively short intervals of 3, 8, and 24 hr would be expected to be much less effective than continuous exposure for 6 days. We examined this [96] and the results are summarized here. This experiment was done by adding DFO to the culture wells then removing it after the specified time by repeated replacement of culture medium. Growth did not recover if the exposure to DFO was $\geq 100 \mu\text{M}$ for ≥ 8 hr; this

short exposure was nearly as effective as the exposure to the same DFO concentration for the full 6 days of the experiment. Although the result was surprising, it is consistent with the lack of improved dose response by constant infusion. We concluded that DFO causes direct and irreversible damage to the parasite.

H. Internal DFO Target in *Pneumocystis* and Mechanism of Action

The obvious question was whether DFO penetrates *Pneumocystis* to cause this damage. DFO is a relatively large molecule, 560 Da for the DFO moiety of the mesylate salt, and has a charged amino group on one end, three polar hydroxamate moieties and two polar amide linkages so that one would not expect it to pass through membranes easily. However, action against the malarial parasite involves penetration of both the host erythrocyte membrane and the parasite membrane [103–105]. Using classical methods for study of transport into cells [96], we were able to detect DFO in *Pneumocystis* exposed to the drug; the amount of drug taken up increased with increasing exposure time. Experiments with the fluorescent vital dye calcein-AM confirmed penetration. This fluorescent vital dye is an iron chelator (less avid than DFO) and the fluorescence is partially quenched by chelation with iron; thus it serves as an indicator of the chelatable iron in the cytoplasm of a cell [106]. DFO caused an increase in fluorescence of cells exposed to calcein-AM, demonstrating both DFO transport and the activity of DFO as an iron chelator within the cytoplasm.

I. Efficacy of Weekly Aerosol DFO Administration

The observed irreversible effect of DFO in our culture system led to consideration that infrequent administration of an adequate dosage could be effective *in vivo*. This was not unprecedented, since Weinberg had reported the results of a single experiment in which mice were treated once weekly with DFO with some effect on PcP. Boelaert et al. [91] confirmed and extended the finding, showing that weekly treatment of a mouse model ($100 \text{ mg DFO kg}^{-1} \text{ IM}$) was very effective.

Efficacy of weekly treatment in the rat model was tested using an aerosol to deliver DFO directly to the alveoli. Preliminary pharmacokinetic studies were performed to design an aerosol dose that would produce the same lung concentration achieved by $1000 \text{ mg DFO kg}^{-1} \text{ IP}$, a dose we had previously shown to be effective if given daily for 3 weeks [87]. This weekly aerosol treatment was remarkably effective both as a prophylaxis and as a curative treatment for animals with fully developed PcP. No *Pneumocystis* cells were found in any of the stained smears from the lungs of treated animals, a degree of *Pneumocystis* clearance we had never observed previously, even with treatment by TMP-SMZ. It must be considered that, although the lung content of DFO via aerosol delivery equaled the content after $1000 \text{ mg kg}^{-1} \text{ IP}$, the transient concentration to which *Pneumo-*

cystis is exposed is no doubt greater with aerosol delivery. A preliminary experiment (unpublished) using weekly 1000 mg kg⁻¹ IP treatment produced about a 50% reduction in parasitosis. Although this is a weak response relative to that obtained by aerosol treatment, it confirms that infrequent treatment has an effect on PcP.

J. Current Status of DFO and *Pneumocystis* Pneumonia and Future Directions

DFO is very effective in animal models of PcP. The dose and frequency required for effective treatment suggests that it could be useful clinically. People with AIDS often respond to drugs differently than other patients and some ordinarily well-tolerated compounds cause severe problems, but this seems not to be the case with DFO. Anecdotal and controlled data suggest that DFO is active against PcP in AIDS patients. In the early 1980s, before there was a test for screening donated blood for HIV, a group of twelve β -thalassemia patients at the Cooley's Anemia Clinic of Cornell University Medical College became infected due to the repeated transfusions required to treat their thalassemia. Of these, only one had an episode of PcP, a patient who did not comply with prescribed DFO therapy (Robert W. Grady, personal communication). This was before PcP prophylaxis was routine and at least 70% of AIDS patients had one or more episode of PcP. An Italian study with β -thalassemia patients who had also become infected with HIV from transfusion showed that those maintained on a higher dose of DFO progressed toward AIDS more slowly than those maintained with less DFO [107]. While there was no explanation of the factors defining AIDS (direct inquiry was made but such data was not collected), historically PcP was one of the major such factors and it may have been that DFO treatment was involved in producing this delay. These results also suggest that treatment with DFO is not injurious for persons infected with HIV. In all of our studies with the rat model of PcP and DFO, we routinely noted that the appearance of DFO-treated rats improved within 24 to 36 hr after treatment is initiated, whereas this does not occur with TMP-SMZ until after 4 or 5 days of treatment (unpublished observation). Because DFO has been demonstrated to have anti-inflammatory properties [108], it is possible that this rapid improvement was due to a combination of specific therapy combined with symptomatic relief due to anti-inflammatory action. This would be similar to the current practice of including steroid therapy with initial treatment for severe PcP.

Other than the action of DFO being internal, we have no information on the underlying molecular basis of the interaction of DFO and *Pneumocystis*. There is more information for malaria. Continuous exposure to low concentrations of DFO is effective against *Plasmodium*, but high intermittent exposure is not [109,110], the opposite of the situation with *Pneumocystis*, thus suggesting a

different mode of action. However, the effect of DFO on *P. falciparum* is internal and can be irreversible [70], suggesting that mechanisms could be similar. In malarial culture, the iron content of serum used in the medium is unrelated to the efficacy of DFO in preventing growth of this protozoan [111]. The same is true in a rat model of malaria, in which the effect of DFO on parasitemia was independent of host iron status [112]. These observations suggest that reducing iron in the parasite milieu has no effect on growth. Independence of the iron supply and requirement for direct penetration means that the mechanism of action is of DFO is not by deprivation of nutritional iron in the classical sense. On the other hand, a new definition of nutritional deprivation has been proposed that would include penetration by a chelator with subsequent removal of iron depriving the microbe of iron needed for cellular function [69]. Supporting this is work with compounds termed shuttle chelators [113]. These easily penetrate cells and have high iron affinity but not as high as that of DFO. They are thought to operate by shuttling iron from the cytoplasm to the extracellular milieu, where they can be bound by the very high affinity chelator DFO, thus creating a faster net flow of iron out of the target cells [114]. Shuttle chelators have been shown to be synergistic with DFO against *Plasmodium* [115] and could be synergistic with DFO against *Pneumocystis*.

The following questions are approachable but as yet unanswered. Does DFO extract iron from *Pneumocystis*, as seems to be the case for *Plasmodium*? Alternatively, does the DFO iron complex, which our data indicates forms within *Pneumocystis*, remain within the fungus? Can shuttle chelators improve the activity of DFO? Does DFO simply bind to iron within the cytoplasm, making it unavailable for cell function and thus creating an internal nutritional deficiency? Or, instead, does DFO remove iron from critical enzymes or complex to an iron-containing enzyme interfering with function? What changes in metabolic processes occur after exposure to DFO? What changes in protein expression does *Pneumocystis* make after exposure to DFO? These questions await further research.

List of Abbreviations

AdoHcy: S-adenosylhomocysteine

AdoMet: S-adenosylmethionine

AdoMetDC: S-adenosylmethionine decarboxylase

BBS: MDL 27695 or (*N,N'*-bis{3-[(phenylmethyl)amino]propyl}-1,7-diaminoheptane or $C_6H_5CH_2NH(CH_2)_3NH(CH_2)_7NH(CH_2)_3NHCH_2C_6H_5$)

CHO: Chinese hamster ovary

CoA: coenzyme A

dcAdoMet: decarboxylated AdoMet

DFMO: DL- α -difluoromethylornithine

DFO: desferrioxamine, deferoxamine, Desferol

FAD: flavin adenine dinucleotide
FDA: U.S. Food and Drug Administration
Hcy: homocysteine
HPLC: high-performance liquid chromatography
HT115: human colon carcinoma cell line
 K_m : Michaelis-Menten constant
L1210: mouse leukemia cell line
MGBG: methylglyoxal bis(guanylhydrazine)
ODC: ornithine decarboxylase
PAO: polyamine oxidase
PcP: *Pneumocystis* pneumonia
 P_i : inorganic phosphate
 PP_i : pyrophosphate
SHAM: salicylhydroxamic acid
SMZ: sulfamethoxazole
SSAT: spermidine/spermine-N¹-acetyl transferase
TMP: trimethoprim
TMP-SMZ: trimethoprim = sulfamethoxazole

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23

DHFR and IMPDH: Enzymes Exploitable as Drug Targets in *Pneumocystis*

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I. Introduction

Many classes of drugs have been tested against infections caused by *Pneumocystis* since the early part of the twentieth century, when the organism was first shown to cause disease. The two most effective therapies identified empirically were the antifolates sulfamethoxazole-trimethoprim in fixed combination and the anti-protozoal diamidine pentamidine. The principal of selective toxicity requires that drugs be targeted to block required processes in pathogenic organisms and that the targeted process be different enough so that the host is unharmed while growth of the pathogenic organism is prevented or the organism is even destroyed. Thus, improvement over these empirically selected agents requires robust biochemical support both to isolate the drug target and to evaluate inhibition of the target in the intact organism. *Pneumocystis* does not easily lend itself to biochemical study, but in the last few years methods for isolating purified and biochemically active organisms have improved [1–9]. These methods, coupled with the powerful methods of recombinant DNA technology, have facilitated studies directly on drug targets in the organism [10–12].

This chapter is restricted to dihydrofolate reductase (EC 1.5.1.3), a key enzyme of the folate pathway, and to inosine 5'-monophosphate dehydrogenase

(EC 1.1.1.205), a key enzyme in purine metabolism. Dihydropteroate synthase, another key folate pathway enzyme that serves as the target for sulfonamides, is covered in Chap. 23.

II. Dihydrofolate Reductase (DHFR)

A. Rationale for Use as a Drug Target

Because the empirical drug therapy for *Pneumocystis* infections had included antifolates, the enzymes of the folate pathway were naturally the first enzymes from the organism to be studied in detail. De novo folate synthesis in rat-derived *Pneumocystis* was confirmed by Kovacs et al.[4]. Known inhibitors of DHFR reduced counts of rat-derived organisms in a short-term culture system, with the lipid-soluble compounds trimetrexate and piritrexim being much more potent than trimethoprim [13]. DHFR inhibitors were noted to have weak activity in the most widely used rat model for *Pneumocystis* pneumonia, but the failure may be related to differing pharmacokinetics in rodents vs. humans [14]. Certainly clinical experience confirms that DHFR inhibitors are key elements in successful management for most patients; trimethoprim or pyrimethamine are common components of chemoprophylaxis regimens, whereas trimethoprim or trimetrexate are used in therapy regimens [15].

DHFR isolated from rat-derived *Pneumocystis* was first characterized by Kovacs et al. [2] and shown to be a small 26-kDa protein that purified independently of thymidylate synthase. This information was useful in supporting RNA sequence data suggesting that *Pneumocystis* was more closely related to fungi than to protozoans; protozoans possess a large bifunctional protein containing both DHFR and thymidylate synthase activity, whereas fungi possess two separate proteins. More importantly, the isolated enzyme was subject to inhibition by a variety of compounds known to inhibit DHFR activity in other organisms [1,16–18].

B. Sources of DHFR for Study

The earliest studies cited above used DHFR isolated directly from *Pneumocystis* organisms, which in turn had to be isolated from rat lungs by laborious methods including differential disruption, sieving, and/or gradient centrifugation. The yield of enzyme was at best about 58 IU per rat (1 IU = 1 nmol of NADPH oxidized per min) [1]. Clearly, recombinant enzyme was necessary to support the enzyme characterization and inhibitor studies that needed to be done.

The first report of recombinant DHFR from *Pneumocystis* came from Edman et al. [10], who used the differential sensitivity of *Escherichia coli* and *Pneumocystis* DHFR to trimethoprim as a selection method to isolate cDNA for *Pneumocystis* DHFR from a lambda phage library. The cDNA that represented

the DHFR sequence from organisms originally isolated from rat lungs was used to show that the DHFR gene was on a different chromosome than the gene for thymidylate synthase; it was also used as a probe against genomic DNA from five rat and two human isolates of *Pneumocystis*. (The organism isolated from human samples is hereafter referred to as *Pneumocystis jiroveci*.) These studies showed that all of the sources of DNA gave a product that was identical in the coding region for the protein and all contained a single 43-bp intron. The predicted molecular weight of the protein was 23,868 (206 amino acids).

Later studies have suggested that DHFR sequences from rat-derived *Pneumocystis* and *P. jiroveci* are not identical. Edman et al. [10] had examined two samples of presumably *P. jiroveci*, but Ma et al. examined DHFR sequence data from 37 samples of *P. jiroveci* gathered from patients over a 13-year period [19]. Using primers based on the DHFR from rat-derived *Pneumocystis*, Ma et al. isolated a 597-bp fragment from *P. jiroveci* genomic DNA and showed the fragment was related to DHFR. Subsequent inverse Polymerase chain reaction (PCR) yielded the 3' and 5' ends of the gene. The final product was 1616 bp (GenBank accession number AF090368), and coded for a 206-amino acid protein of predicted molecular weight of 23,407 kDa. The sequence showed an intron in the same position as in the rat-derived *Pneumocystis* DHFR, but the intron was 42 rather than 43 bp.

The most interesting revelation from the work of Ma et al. [19] is that the DHFR from *P. jiroveci* was about 38% divergent in amino acid sequence from the rat-derived enzyme. The question that immediately arises from these results is whether the body of work on DHFR from rat-derived *Pneumocystis* has applicability to drug discovery for the infection in humans. Two analyses are important in trying to answer this question. First, the epidemiology of the infection needs to be clarified. Takahashi et al. examined DHFR nucleotide sequences and deduced amino acid sequences from *P. jiroveci* isolates taken from 27 immunosuppressed patients in Japan [20], using a set of primers reported by Ma et al. [19] to be specific for the DHFR from *P. jiroveci*. With these primers, Takahashi et al. found *Pneumocystis* DHFR sequences in the samples from all patients tested, but 16 of their 27 patients had nucleotide substitutions in the coding region for DHFR; two of these changes cause amino acid substitutions in the protein, but the amino acid changes were judged to be in noncritical regions [20]. Two patients apparently carried a mixture of wild-type and mutant organisms.

Unfortunately the study of Takahashi et al. [20] did not include an attempt to amplify sequences from the bronchoalveolar fluid samples using primers that would have recognized other forms of *Pneumocystis* DHFR—e.g., primers FR01 and FR634 from Ma et al. [19]. Older studies have pointed to heterogeneity in organisms isolated from patients [21–29] as well as from laboratory rats [30–32]. This heterogeneity was confirmed in a careful anatomical study on the lung from

a single patient, where two types of *P. jiroveci* were found, and the types seemed to have a differential distribution in the lung [33].

The results of these epidemiological studies to date suggest that although a prototype organism, *P. jiroveci*, can be identified, considerable heterogeneity is possible. Furthermore, in vitro and in vivo models using either *P. carinii* f. sp. *ratti* or *P. carinii*, the two forms restricted to rats, have accurately predicted the efficacy of many clinically effective agents, including trimetrexate [13,34]. Thus, it may well be premature to limit study to a single form of the organism, but it is certainly important to clarify the genetic identity of the forms of *Pneumocystis* currently used in the various laboratories and, where possible, drug studies should involve multiple forms of the organism. Unfortunately, animal models are not readily available for drug testing of *P. jiroveci* because of the host-species specificity of the organism.

The second issue raised by the discovery of a divergent DHFR from *P. jiroveci* is whether the differences in amino acid sequence cause significant differences in the properties of the protein. This question is important because a large body of data exists on antifolates in tests against *P. carinii* and *P. carinii* f. sp. *ratti*, as well as in vitro tests against both the native and the cloned DHFR from rat-derived organisms. Two laboratories have reported work with native DHFR isolated from *Pneumocystis* from rat lungs [1,2]. The Michaelis constant (Km) values for DHFA and NADPH from the two laboratories agree within statistical error (Table 1). The Km for DHFA for the recombinant form of the rat-derived *Pneumocystis* enzyme also shows agreement between laboratories, but the results suggest that the recombinant enzyme tends to have a lower Km for DHFA than the native enzyme; this difference may reflect the way in which the native enzyme had to be purified from contaminating proteins in whole cell

Table 1 Comparative Kinetics of DHFR from Various Forms of *Pneumocystis*

Source of DHFR ^a	Km for DHFA Micromolar	Km for NADPH Micromolar	Reference
<i>Pneumocystis</i> , rat-derived			
Native	17.6 ± 3.9	40.2 ± 16	2
	10.6 ± 0.8	16.7 ± 1.9	S. Queener, unpublished
Recombinant	1.77 ± 0.23	1.38 ± 0.16	35
	2.5	8.9	17
	4.9 ± 1.1	12.3 ± 2.0	S. Queener, unpublished
<i>Pneumocystis</i> , human-derived (<i>P. jiroveci</i>)			
Recombinant	2.7 ± 0.3	14.0 ± 4.3	36

^a DHFA is the substrate for DHFR; NADPH is a required cofactor for the reaction.

extracts. The K_m for NADPH for recombinant rat-derived *Pneumocystis* DHFR was reported as 1.38 ± 0.16 by Sirawaraporn et al., but two other laboratories report values closer to those seen with native *Pneumocystis* enzyme (Table 1). The single report of kinetic constants for recombinant DHFR derived from *P. jiroveci* yields values indistinguishable from the recombinant enzyme from rat-derived *Pneumocystis* [36]. Thus, there is no clear-cut evidence of kinetic differences between the two forms of the enzyme.

A more stringent test of the degree of similarity between the two forms of DHFR is in their response to an array of well-characterized inhibitors. Four compounds have been used by several laboratories to characterize DHFR from *Pneumocystis*: trimethoprim, pyrimethamine, trimetrexate, and methotrexate (Fig. 1). Trimethoprim is an obvious choice because of its clinical use for pneumonia caused by *P. jiroveci*; but trimethoprim was developed as an antibacterial agent and is the drug is most selective against bacterial forms of DHFR. Pyrimethamine is also used clinically for *Pneumocystis* pneumonia, but its most common application is as an antiprotozoal drug. Trimetrexate and methotrexate are anticancer drugs with strong activity against mammalian DHFR, although trimetrexate is an alternate drug used clinically for therapy of *P. jiroveci* infections[15]. These four DHFR inhibitors are good reference compounds because they show a wide range of potency. IC_{50} values for these four compounds are available for both

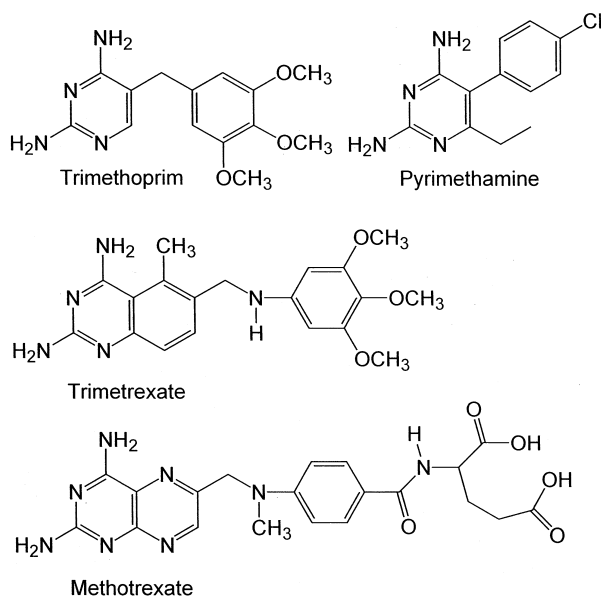


Figure 1 Standard inhibitors of DHFR.

Table 2 IC₅₀ Values for Standard DHFR Inhibitors

Standard Compounds	Rat-Derived <i>Pneumocystis</i> DHFR		DHFR from <i>P. jiroveci</i>
	Native Micromolar IC ₅₀ (Ref.)	Recombinant Micromolar IC ₅₀ (Ref.)	Recombinant Micromolar IC ₅₀ (Ref.)
Trimethoprim	40 (16) 12 (1)	40 (35) ^a 26.8 ± 1.7 ^b	4.85 ± 0.21 (36)
Pyrimethamine	2.8 (16) 3.5 (1)	1.2 (35) ^a 2.4 ^b	0.24 ± 0.03 (36)
Trimetrexate	0.026 (16) 0.042 (1)	0.07 (35) ^a 0.042 ± 0.013 ^b	0.0075 ± 0.0027 (36)
Methotrexate	0.0013 (1)	0.03 (35) ^a 0.001 (37)	0.00062 ± 0.00013 (36)

^a IC₅₀ values were determined at 20 μM DHFA. Others were at 90 to 115 μM DHFA.

^b Queener, unpublished data.

native and recombinant rat-derived *Pneumocystis* DHFR and for DHFR from *P. jiroveci* (Table 2). Agreement of IC₅₀ values is good for both forms of rat-derived *Pneumocystis* DHFR. For example, the IC₅₀ values for trimethoprim range from 12 to 40 μM, only about a threefold range for data from three separate laboratories and over a several-year time span.

Comparison of IC₅₀ values from recombinant rat-derived *Pneumocystis* DHFR and the recombinant DHFR from *P. jiroveci* suggests that some systematic differences may exist (Table 2). For example, the IC₅₀ value for trimethoprim is 2.5- to 8-fold less for the recombinant enzyme from *P. jiroveci* than for the enzyme from rat-derived organisms. IC₅₀ values for the other three compounds are also lower. In contrast, the dissociation constant (K_i) values tend to be slightly higher for the DHFR from *P. jiroveci*, the exception being the K_i for trimetrexate (Table 3).

Table 3 K_i Values for Standard Inhibitors

Inhibitor	Recombinant DHFR from	
	Rat-derived <i>Pneumocystis</i> , K _i , nM	<i>P. jiroveci</i> K _i , nM
Trimethoprim	152 ± 10	280 ± 80
Pyrimethamine	9.7 ± 0.3	65 ± 5
Trimetrexate	0.71 ± 0.04	0.23 ± 0.03
Methotrexate	0.0081 ± 0.0005	0.016 ± 0.004

Sources: Data in column 1 are from Ref. 17; data in column 2 are from Ref. 36..

The IC_{50} data would lead to the conclusion that, at saturating substrate and cofactor concentrations, the DHFR from *P. jiroveci* would be more sensitive to standard drugs than the DHFR from rat-derived organisms; the K_i data would suggest the opposite. For example, achievable serum concentrations with trimethoprim range around 3.1 $\mu\text{g/mL}$ (about 11 μM) in human patients, and the drug penetrates reasonably well to many body compartments. Knowing that trimethoprim is a competitive inhibitor [36] and accepting a K_m for DHFA of about 3 μM (Table 1), we can calculate relative inhibition of DHFR at 11 μM trimethoprim and various concentrations of DHFA, using the standard equation for competitive inhibition: $v/V_{\text{max}} = [S]/([S] + K_m\{1 + [I]/K_i\})$. By this calculation, at 100 μM DHFA (i.e., saturating substrate concentration) the DHFR from rat-derived organisms would be 70% inhibited, but the enzyme from human-derived organisms would be 55% inhibited. At 10 μM DHFA (i.e., limiting substrate concentration), the values would be 96% inhibition for DHFR from rat-derived organisms and 80% inhibition for DHFR from human-derived organisms. These differences with known inhibitors are relatively small and suggest that, at clinically achievable concentrations of trimethoprim, both forms of the enzyme will be significantly inhibited.

Analysis of either the IC_{50} values or the K_i values also suggests that the order of potency of these four reference compounds is the same for studies using native or recombinant DHFR from rat-derived organisms or recombinant DHFR from *P. jiroveci*. So far, any drug judged potent in assays against one form for DHFR has also been potent against the other form.

C. Assessing Selectivity

Another issue facing those interested in antifolate drug discovery for *Pneumocystis* is selectivity of compounds. For example, methotrexate is one of the most potent DHFR inhibitors ever tested against the enzyme from rat-derived *Pneumocystis* or *P. jiroveci* (Tables 2 and 3). The drug is not used clinically to treat *Pneumocystis* infections because it is not selective—i.e., it is as toxic to the patient as to the pathogen. In an attempt to evaluate drug candidates for selectivity as well as for potency, the original NIH-funded contract for drug discovery of anti-*Pneumocystis* drugs called for side-by-side comparisons of in vitro activity of DHFR inhibitors using not only enzyme from the pathogen but also DHFR from a mammalian source [1]. The original choice of rat liver DHFR as the reference enzyme was driven by ease of preparation, abundance, and cost; scant data existed at the time to suggest that any one mammalian DHFR would be significantly different from another. Today, x-ray crystallographers are able to examine the active sites of many forms of DHFR and to evaluate the potential differences. The cloning of rat liver DHFR has recently been reported [37], and

studies are under way to compare that enzyme to human DHFR (V. Cody, personal communication, 2002).

In the absence of direct x-ray data, we can still compare studies that used rat liver DHFR with those that used human DHFR to see whether conclusions about selectivity of drug candidates were similar with the two types of reference enzymes. Data from four studies are assembled in Table 4, comparing the dimensionless selectivity index, which is defined as the ratio of IC₅₀ value for the mammalian reference DHFR to the IC₅₀ value for the DHFR from the pathogen [1]. The values in Table 4 show that for all forms of mammalian reference enzyme, the selectivities are very similar for any one drug; selectivities vary less than twofold across the table for trimethoprim or for methotrexate. Pyrimethamine shows the greatest variance, but values determined with the recombinant rat-derived *Pneumocystis* DHFR are very similar (Table 4, last three columns). More importantly, the order of selectivity is the same in these studies: trimethoprim is most selective, followed by methotrexate, then pyrimethamine, with trimetrexate being least selective. The only exception is in one study with native rat-derived *Pneumocystis*, where the selectivity of pyrimethamine and methotrexate are essentially the same (Table 4, column 1). A similar analysis of selectivity can be performed using published Ki values for these compounds determined using recombinant rat-derived *Pneumocystis* DHFR and recombinant human DHFR as a reference enzyme [17]. In this study, the order of selectivity is identical to that derived from the studies in Table 4: trimethoprim > methotrexate > pyrimethamine > trimetrexate.

The conclusions to be drawn from these analyses are that at present there is no evidence of strong systematic differences between rat liver and human DHFR in terms of either potency or selectivity for reference compounds; nevertheless, there is no reason not to move forward with comparisons between the two

Table 4 Selectivity for Standard Inhibitors Assessed with Human or Rat Liver DHFR

Inhibitor	Selectivity index			
	ratIC50/ pcIC50 (1)	ratIC50/ RpcIC50 (37)	RratIC50/ RpcIC50 (37)	RhumIC50/ RpcIC50 (37)
Trimethoprim	11	11	10	16
Pyrimethamine	2	0.3	0.3	0.6
Trimetrexate	0.07	0.19 ^a	—	—
Methotrexate	1.9	1.1	1.1	1.4

^a Queener, unpublished data.

Key: Rhum, recombinant human DHFR; rat, native rat liver DHFR isolated biochemically; Rrat, recombinant rat DHFR; Rpc, recombinant rat-derived *Pneumocystis* DHFR; pc, native DHFR from *Pneumocystis* isolated from rat lungs.

reference enzymes. The ready availability of the recombinant human enzyme overcomes logistical barriers to using human DHFR as one standard. For continuing studies within single laboratories with long-standing development programs, there may be advantages to using both rat liver and human DHFR for a period of time until x-ray crystallography clarifies how these two forms of enzyme interact with key experimental compounds and to allow historical comparisons to be made to previous compounds within a series.

D. Current Research Directions Exploiting DHFR as a Drug Target

Several laboratories have long-standing programs of drug discovery for antifolates acting upon DHFR from *Pneumocystis*. The body of work up until 1995 has been reviewed [38]; therefore this presentation focuses on work done since that time and primarily considers studies where x-ray crystallography has consolidated the conclusions drawn from the in vitro enzyme data. The work on rotational analogues of pyrimethamine and related compounds has also recently been reviewed [39] and is not considered here. The ideal DHFR inhibitor being sought in all these studies is one that combines the potency of trimetrexate or methotrexate with the selectivity of trimethoprim. The compounds discussed below are all more selective than trimethoprim and considerably more potent.

One of the most interesting recent leads is compound PT653 (Fig. 2), created in a structure-based design program involving the laboratories of Andre Rosowsky [40] and Vivan Cody [41]. This compound is over 50-fold more potent than trimethoprim (IC_{50} for trimethoprim/ IC_{50} for PT653 = 12 μ M/0.21 μ M = 57) against the recombinant DHFR derived from *Pneumocystis* from rats (Table 5). Along with this increased potency, a nearly twofold increase in selectivity was noted. This compound built on earlier studies by Piper et al., who also evaluated analogues of this type with bulky substituents at the 6 position [42]. PT653 was considered of interest for x-ray crystallographic studies because of its high selectivity and the questions posed by the bulky substituent at the 6 position. Accordingly Cody et al. studied the structure of recombinant rat-derived *Pneumocystis* DHFR in ternary complex with PT653 and NADPH, the cofactor for the DHFR reaction [43]. The hypothesis this study explored was whether the bulky N,N-diarylaminomethyl substituent at the 6 position of the 2,4-diaminopteridine ring in compound PT653 could be shown to fit better into the active site of the *Pneumocystis* DHFR than it could into the active site of mammalian DHFR. Two very interesting conclusions were drawn from this study. First, PT653 induces a conformational change in the enzyme, causing a large movement of flexible loop 47 of the structure, which is formed by residues 45 to 50; induced conformational shifts in this region had been seen earlier for folate [44]. Second, PT653 binds with a different orientation of the pteridine ring system, tilting slightly from the orientation seen with methotrexate and other classical antifolates. This tilt

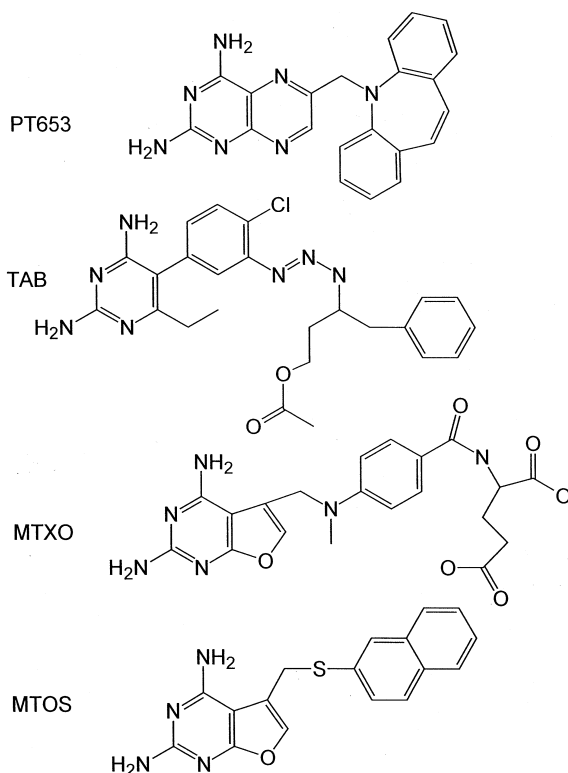


Figure 2 Experimental DHFR inhibitors.

Table 5 Experimental DHFR Inhibitors Showing Potency and Selectivity Toward Rat-Derived *Pneumocystis* DHFR

Compound	Reference	Designation in reference	IC ₅₀ Values, μ M		Selectivity index
			RpcDHFR	ratDHFR	
PT653	40	1	0.21	4.4	21
TAB	46	14a	0.17	19.4	114
MTXO	48	2	0.035	0.43	12
MTOS	50	5	0.65	12.3	19

Key: RpcDHFR, recombinant DHFR from rat-derived *Pneumocystis*; ratDHFR, native rat liver DHFR.

maintains hydrogen bonding, which stabilizes the pteridine ring in the active site but also creates a better orientation for the bulky hydrophobic substituent at the 6 position. The result is a unique fit into the roomy active site of *Pneumocystis* DHFR, one that is not well accommodated by the smaller mammalian DHFR active site.

PT653 has been tested in two assays against intact organisms. Preliminary results of an uptake study show that 18 μ M PT653 caused 62% inhibition of uptake of p-aminobenzoic acid by *P. carinii* freshly isolated from rat lung (Queener, unpublished data). The compound was also tested against *Saccharomyces cerevisiae* that had been modified by having the native DHFR gene replaced by the coding sequence of the DHFR from rat-derived *Pneumocystis* [45]. Although trimetrexate and a limited number of other antifolates showed some activity in this yeast model system, PT653 was inactive against the recombinant yeast containing *Pneumocystis* DHFR, although it did show activity against the yeast reconstituted with DHFR from *Plasmodium falciparum*. The full potential of this compound and its analogues has yet to be fully explored in animal models.

Another interesting antifolate, TAB, is an analogue of pyrimethamine with a triazenyl substituent ortho to the chlorine [46]. Compounds of this general type were first noted to be selective in early work with native rat-derived *Pneumocystis* DHFR [1]. TAB has a potency similar to that of PT653 but is less effective against the rat liver reference enzyme, with the result that the selectivity is 114 [46]. This selectivity is the highest noted in this in vitro model, being about tenfold more selective than that of trimethoprim. Molecular modeling studies and a crystal structure revealed that TAB interacts with the active site of recombinant DHFR from rat-derived *Pneumocystis* in at least two orientations [47]. Although the compound binds in general like trimethoprim or methotrexate, rotation around the N2–N3 bond in the triazenyl substituent is possible (Fig. 2). One of these orientations places a methyl group and the benzyl group in contact with large hydrophobic regions of the folate binding pocket. In particular, a phenylalanine at position 69, which is unique to the DHFR from rat-derived *Pneumocystis*, may be involved in this interaction. Thus this compound takes advantage not only of the larger hydrophobic area in the active site of the rat-derived *Pneumocystis* DHFR but also seems to take advantage of a unique residue.

Furo[2,3-d]pyrimidines have been explored for both their potency and selectivity. Gangjee et al. synthesized a lead compound, MTXO, in 1994 [48], which was over 700-fold more potent than trimethoprim and about as selective (Table 5). The binding of this compound to recombinant DHFR from rat-derived *Pneumocystis* was studied, using binding to human DHFR for comparison [49]. Three differences were noted. First, when rat-derived *Pneumocystis* DHFR bound MTXO, an interaction was allowed between the inhibitor and the nicotinamide cofactor; this interaction was not observed in the human DHFR. Second, an additional hydrogen bond was generated with the conserved tyrosine 129 and

MTXO, but this interaction was not seen in the human DHFR, although the analogous tyrosine residue is present. Third, the unique phenylalanine at position 69 interacts with MTXO when bound to the rat-derived *Pneumocystis* DHFR but not the human enzyme. These interactions differ from those seen with methotrexate and may explain the lower activity of MTXO as well as its selectivity.

Another furo[2,3-d]pyrimidine of interest was also created by Gangjee et al. [50]. This compound, referred to as MTOS, bears a sulfur in the side chain linking the furo-pyrimidine ring to a 2-naphthyl group. The potency of the compound is superior to that of trimethoprim or pyrimethamine and its selectivity is about the same as that of PT653 (Table 5). MTOS binds to the active site of *Pneumocystis* DHFR in a manner similar to that of MTXO; like MTXO, MTOS interacts with the phenylalanine at position 69 of the rat-derived *Pneumocystis* enzyme, thus stabilizing a hydrophobic interaction.

The structural studies with the lead compounds discussed above point out that several important interactions involve the presence of a larger active site in the enzyme from rat-derived *Pneumocystis* relative to the human enzyme; the ligand-induced conformational shifts in at least one flexible loop (residues 45 through 50), which is largely missing in the human enzyme; and interactions with phenylalanine 69 in the rat-derived *Pneumocystis* enzyme. Because the sequence of the DHFR from *P. jiroveci* differs significantly from that of rat-derived *Pneumocystis* DHFR, it is important to compare the two proteins in some of the regions noted to be important for the drug interactions. First, both forms of *Pneumocystis* DHFR are of the same size, so that the flexible loop structure would seem likely to be preserved [19]. Likewise, the regions involved in forming the active site and in particular the folate binding region are predicted to be similar [19]. One substantial difference between the two sequences is that the rat-derived *Pneumocystis* DHFR contains a phenylalanine at position 69; a phenylalanine at this position is also seen in the enzyme from *Candida albicans*, *Toxoplasma gondii*, and *Plasmodium falciparum* but is absent from the DHFR from rat, mouse, Chinese hamster, human, or *P. jiroveci* [37]. Because of this difference and the potential role the residue may play in inhibitor interaction, it will be crucial to test compounds such as TAB, MTXO, and MTOS against DHFR from *P. jiroveci* and from the rat forms *P. carinii* and *P. carinii* f. sp. *ratti* to assess the role of phenylalanine 69 in producing potency and selectivity. These compounds would also seem to be good candidates to assess potential differences in inhibitor potency between the two enzymes.

In addition to the design strategies noted above, based on the hypothesis that a highly selective DHFR inhibitor can be designed that will be appropriate for systemic administration to treat *Pneumocystis* pneumonia in humans, other approaches are being considered. For example, Gangjee and his group have explored dual DHFR and thymidylate synthase inhibitors [51]. A Scandinavian group is exploring the possibility of designing ester analogues of active com-

pounds [52]. This so-called soft drug approach involves local administration of drugs designed to act at the site of administration and then be quickly metabolized to inactive compounds before being distributed throughout the body. Neither of these strategies has yielded drug candidates with improved potency and selectivity over standard compounds to date but work continues.

III. Inosine 5' Monophosphate Dehydrogenase (IMPDH)

A. Rationale for Use as a Drug Target

IMPDH has attracted attention in recent years as a drug target with remarkable versatility. Inhibitors of mammalian forms of this enzyme are used clinically as immunosuppressants and are being explored as anticancer agents [53–55]. Inhibition of the enzyme also interferes with viral reproduction, including that of HIV [56,57]. An inhibitor of plasmodial IMPDH has been investigated as an antimalarial drug [58]. The attractiveness of IMPDH as a drug target derives from the cross-species diversity of the enzyme and the key position this enzyme holds in purine metabolism.

IMPDH is usually considered to be the rate-limiting step in guanine biosynthesis, although studies to confirm this role have not been carried out in all organisms of interest. De novo synthesis produces IMP as a key intermediate, which can then be converted to either AMP and its products of GMP and its products (Fig. 3).

IMPDH may also play a role in salvage mechanisms to produce purines. In theory, organisms might take up adenine, hypoxanthine, xanthine, guanine, adenosine, inosine, or guanosine to supply their needs for adenine or guanine nucleotides. If adenine, adenosine, inosine, or hypoxanthine are the only compounds available or the only ones capable of being salvaged, a functional IMPDH is required to supply guanine nucleotides to the organism.

The only enzyme shown in Figure 3 that has been directly proven to exist in *Pneumocystis* is IMPDH. The gene for the enzyme has been identified and sequenced, mRNA has been detected, and a functional enzyme has been cloned from cDNA derived from the mRNA [59,60]. There is also strong circumstantial evidence that the enzyme is required for growth of *Pneumocystis* both in vitro and in vivo. In vitro studies using a short-term culture method that is predictive for drug susceptibility with rat-derived *Pneumocystis* showed that mycophenolic acid, a known inhibitor of IMPDH from eukaryotic organisms, was an extremely potent inhibitor of *Pneumocystis* growth [59,60]. Inhibition of growth was nearly complete at 1 μ M concentrations of mycophenolic acid, suggesting that inhibition of IMPDH alone was sufficient to impair purine metabolism to the point of preventing growth. Attempts to reverse the growth inhibition with guanosine or guanine were unsuccessful, which also suggests that salvage of these components

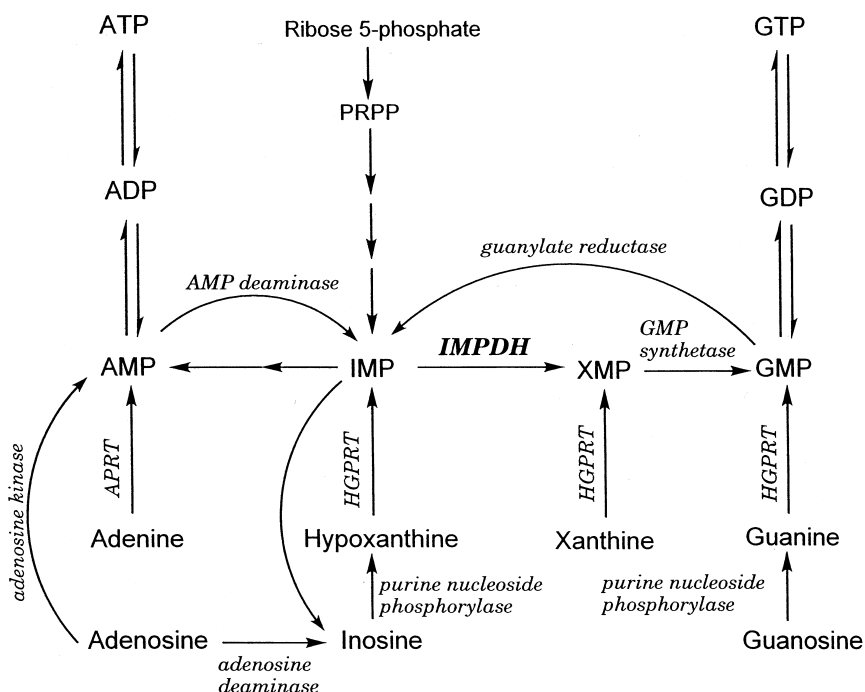


Figure 3 Purine metabolic pathways. PRPP, phosphoribosyl-pyrophosphate; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine, guanosine phosphoribosyltransferase.

was weak or absent. Few modern studies exist on uptake of relevant compounds, but one early study suggested that neither hypoxanthine nor thymidine was taken up by rat-derived *Pneumocystis* organisms [61]. The enzyme that would presumably allow salvage of hypoxanthine, xanthine, or guanine is HGPRT (hypoxanthine, guanosine phosphoribosyltransferase, EC 2.4.2.8), but this enzyme has not been identified in *Pneumocystis*.

The strong action of mycophenolic acid against *Pneumocystis* was seen not only in the short-term culture model but also in an animal model of *Pneumocystis* infection. Oz and Hughes reported that when tested as an immunosuppressant in a rodent model for *Pneumocystis* pneumonia, mycophenolic acid in fact exhibited strong protective effects, preventing infection of the animals [62]. Clinical data also exist for mycophenolic acid, because the drug is used clinically as an immunosuppressant (mycophenolate mofetil, CellCept, Roche). Immunosuppression in patients results from selective action on lymphocytes, which have little purine salvage and are therefore more vulnerable to mycophenolic acid than other mam-

malian cells with more effective salvage mechanisms. The clinical trials of the drug consisted of three groups of patients, one receiving placebo, one receiving 2 g of mycophenolate mofetil and one receiving 3 g of the drug. Opportunistic infections, along with other clinical parameters, were monitored in these patients. Viral infections increased in the two groups of patients receiving mycophenolate mofetil, presumably because they were more immunosuppressed than the placebo group, but the reverse pattern was seen with *P. jiroveci*; there were 4 cases of *P. jiroveci* pneumonia in the 166 patients receiving placebo and none in the 325 patients who received mycophenolate mofetil [63]. Although the number of patients was small and the study was not designed to address the question of differing incidence of opportunistic infections, this study remains an interesting anecdote, which supports the animal and culture studies suggesting that inhibition of IMPDH alone may be sufficient to prevent growth of *Pneumocystis*.

B. Sources of IMPDH for Study

To date, IMPDH has been studied only in *P. carinii* and not in *P. jiroveci*. Our early attempts to assay IMPDH in extracts of *P. carinii* that contained DHFR and dihydropteroate synthase were inconclusive, suggestive of weak activity that was difficult to see above background in crude or partially purified extracts. Although we ultimately succeeded in detecting the enzyme activity in about half of the extracts that showed strong DHFR activity, the activity of IMPDH remained weak, and we thus concluded that recombinant IMPDH would be required for adequate study of this drug target. The cloning strategy has been described elsewhere [59,64]. The original cloning using RNA from cultured rat-derived *Pneumocystis* yielded an mRNA that coded for a putative IMPDH that was 70 amino acids shorter at the N-terminus than other related forms of the enzyme. When the enzyme was recloned using RNA from organisms freshly harvested from rat lungs, several splice variants of the message were noted, but the most abundant message coded for a protein of 529 amino acids, including an additional 75 amino acids at the N-terminus of the protein. This long form of *P. carinii* IMPDH showed strong sequence homology to known forms of the enzyme, especially to those from other fungi [64]. This longer form of the enzyme was also detected in extracts of *P. carinii* using Western blot analysis, suggesting that this is the functional form of IMPDH in vivo in the organism [60].

The catalytic activity of the *P. carinii* IMPDH was confirmed in complementation studies in which the IMPDH-deficient LH3 strain of *E. coli* (kindly supplied by Dr. Liz Hedstrom of Brandeis) was transformed with expression vectors and induced under conditions to allow expression of the recombinant protein. Only those cultures containing bacteria that were able to produce recombinant IMPDH were able to grow in the absence of guanine, suggesting that the recombinant protein was catalytically active. Moreover, this growth was highly

susceptible to inhibition by mycophenolic acid, showing an IC_{50} of $0.17 \mu M$, which is characteristic of the susceptibility predicted for the *P. carinii* enzyme; growth of native *E. coli* is not sensitive to mycophenolic acid, reflecting the relative resistance of the bacterial IMPDH to mycophenolic acid.

The recombinant *P. carinii* IMPDH was expressed in several different forms, including one that contained a his-tag at the N-terminus. Purified preparations of the recombinant protein had a k_{cat} of 0.56 ± 0.032 1/sec when assayed at $25^{\circ}C$ [60]. The *P. carinii* IMPDH was also sensitive to mycophenolic acid, with a K_i value of 60 nM. A preliminary study of recombinant *P. carinii* IMPDH lacking the his-tag has also produced catalytically active enzyme with kinetics similar to that of the his-tagged version of the enzyme.

Production of large amounts of recombinant IMPDH to support biochemical and x-ray crystallographic studies has been achieved, but the vast majority of the protein is recovered in inclusion bodies. Purification of the inclusion bodies followed by solubilization in various concentrations of urea and guanidine yields a soluble protein that is largely inactive in enzyme assays. Because the active form of IMPDH is tetrameric, we must address not only issues of refolding each monomer correctly but also of inducing the refolded monomers to associate to form tetramers. One technique that has yielded active enzyme is a very slow dialysis to remove the 6M urea from the resolubilized protein, ultimately reconstituting the enzyme in 25 mM Tris buffer with 100 mM NaCl. Another protocol has added a reducing agent (20 mM dithiothreitol) to the buffers at each stage and substituted KCl for NaCl; this strategy has also yielded active enzyme. Current studies are aimed at optimizing this refolding/association step.

C. Assessing Selectivity

Human cells can express two different forms of IMPDH. Human type I is 84% homologous to human type II, and although both are susceptible to inhibition by mycophenolic acid, type II is more sensitive [65]. The sequence of the active site of the enzyme is very similar, with no differences in the signature sequence between the IMPDH from *P. carinii* and human type II IMPDH (Fig. 4); the human type I enzyme has a single amino acid change in this region. There is more diversity in the regions immediately flanking the signature sequence. Overall, the human forms of IMPDH are about 65% homologous to the IMPDH from *P. carinii*.

The kinetics of the human forms for IMPDH are similar to those for *P. carinii* IMPDH [60]. Most of the studies on the human enzyme have focused upon the type II enzyme because it was originally considered to be the form that could be induced by cell proliferation, including that of cancer cells. The K_i for mycophenolic acid against type II human IMPDH is similar to the value we determined for *P. carinii* IMPDH. Ideally, both forms of human IMPDH should

[LIVM]-[RK]-[LIVM]-G-[LIVM]-G-x-G-S-[LIVM]-C-x-T

	1	31
Pc	LISAGADALR V GMGSGS I CI TQEIMAVGRP Q	
Ca	LIEAGADALR I GMGSGS I CI TQEVMACGRP Q	
HII	LIDAGVDALR V GMGSGS I CI TQEV L ACGRP Q	
HI	LIDAGVDGLR V GMGCGS I CI TQEVMACGRP Q	
Sp	LIAAGADGLR V GMGSGS A CI TQEVMACGRP Q	
Sc	LIAAGADGLR I GMGSGS I CI TQEVMACGRP Q	
Consensus	LI.AG A DaLR ! GMG s GS i CI TQE ! \$AcGRP Q	

Figure 4 IMPDH/GMP reductase signature sequence homology [LIVM]-[RK]-[LIVM]-G-[LIVM]-G-x-G-S-[LIVM]-C-x-T. Consensus symbols: ! = 1 or V, \$ = L or M, % = F or Y, # = N,D,Q,E,B, or Z. Pc, *Pneumocystis carinii*; Ca, *Candida albicans*; HII, human type II IMPDH; HI, human type I IMPDH; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*. Multalin v. 5.4.1, Copyright I.N.R.A. France 1989, 1991, 1994, 1996.)

be assessed for inhibition as selectivity controls for experimental compounds designed against *P. carinii* IMPDH, but most of the available data are for the type II human enzyme.

D. Current Research Directions Exploiting IMPDH as a Drug Target

Because many analogues of mycophenolic acid and other types of IMPDH inhibitors have been created to be tested as immunosuppressants or as antimicrobial agents, it is possible to screen many compounds in a relatively short time to generate potential leads for a specific drug discovery program. Accordingly, in collaboration with Dr. K. Pankiewicz, we have tested a preliminary series of inhibitors of IMPDH with the purified *P. carinii* IMPDH to discover any differences between the mammalian enzyme and the enzyme from this eukaryotic pathogen. The most dramatic difference is with 7-O-C2-MAD, a derivative of mycophenolic acid adenine dinucleotide. This compound is one of a series designed to resist destruction by common esterases in the body. The 7-O-C2-MAD is inactive against the type II human enzyme but has significant activity against *P. carinii* IMPDH in four independent trials, suggesting considerable selectivity toward the pathogen (Table 6) [60].

These studies suggest that a more extensive array of compounds should be assessed, focusing on analogues of mycophenolic acid but not exclusively limited to them. We are also exploring production of quantities of purified *P. carinii*

Table 6 Mycophenolic Acid Analogues Showing Selectivity for *P. carinii* IMPDH

Compound name ^c	<i>P. carinii</i> IMPDH ^a IC50, μ M	Human IMPDH ^b IC50, μ M	IC50 Ratio, Human/ <i>P. carinii</i>
Mycophenolic acid	0.066 ± 0.003	($K_i = 0.01 - 0.037$)	
C2-MAD	0.063 ± 0.005	($K_i = 0.33$)	
7-O-C2-MAD	6.01 ± 1.55	Inactive	$>29^d$
C6-MAD	79.7 ± 22.3	($K_i = 0.3$)	

^a Recombinant his-tagged long IMPDH assayed under standard conditions, 25°C. IC50s are means $\pm$ SEM for three or four independent assays with separate enzyme preparations.

^b Type II enzyme; data from K. Pankiewicz except for mycophenolic acid (65).

^c All compounds except mycophenolic acid were supplied by K. Pankiewicz.

^d Based on highest concentration tested against the human enzyme.

IMPDH to support x-ray crystallography of mycophenolic acid and its analogues in the active site of the enzyme in order to explore the molecular basis of selectivity and potency. These studies are under way.

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List of Abbreviations

DHFA: dihydrofolic acid, the substrate for DHFR

DHFR: dihydrofolate reductase (EC 1.5.1.3), a key folate pathway enzyme

IC50: inhibitory concentration 50%, the concentration of inhibitor producing 50% inhibition under defined conditions of assay

IMPDH: inosine monophosphate dehydrogenase (EC 1.1.1.205), a key purine pathway enzyme

K_i : the dissociation constant for the enzyme/inhibitor complex

K_m : the Michaelis constant for enzyme and substrate or cofactor

MTOS: an experimental DHFR inhibitor

MTXO: an experimental DHFR inhibitor

PCR: polymerase chain reaction

PT653: an experimental DHFR inhibitor

TAB: an experimental DHFR inhibitor

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Dihydropteroate Synthase and Cytochrome B: The Problem of Emerging Resistance

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I. Introduction

Antifolates and hydroxynaphthoquinones are among the oldest known antimicrobial agents. The prototype antifolate was prontosil, discovered by Gerhard Domagk in the early 1930s. Prontosil proved to be a prodrug, activated by host metabolism into a sulfonamide. Various other sulfonamides were proven to be effective antimicrobials by the late 1930s. The first clue about their mechanism of action was the observation that p-aminobenzoic acid antagonized their antibacterial activity, published in 1940. In 1962, it was shown to act via the inhibition of dihydropteroate synthase (DHPS), an early enzyme in folate biosynthesis (reviewed in Ref. 1). Dapsone, a sulfone, also acts on this target and was first developed in the 1940s to treat leprosy [2].

A second group of antimicrobials act by inhibiting dihydrofolate reductase (DHFR), another enzyme in folate biosynthesis. These were developed by Hitchings, Elion, and collaborators, who synthesized pyrimethamine and trimethoprim as structural analogues of methotrexate in the 1950s [1,3]. Cotrimoxazole, a combination of trimethoprim and sulfamethoxazole, was first marketed in the 1960s. This was the first fixed combination of two antibiotics; it was developed because the two compounds were synergistic and the combination would delay resistance [4].

The hydroxynaphthoquinones became important when they were synthesized and found to have antimalarial activity by a consortium of scientific groups attempting to develop new antimalarials during World War II [5]. The best compound, lapinone, was soon found to act by inhibiting respiration in malaria parasites, but was discarded because it was active only parenterally [2]. In the 1980s and 1990s, an orally active derivative, atovaquone, was developed and found to be highly effective against both malaria and *Pneumocystis carinii* infections [6]. The target of these compounds in malaria was found to be the cytochrome bcl complex in the mitochondrial electron transport chain [7].

II. Sulfa Drugs and DHPS Polymorphisms in *Pneumocystis carinii*

Sulfamethoxazole, a sulfa drug, and dapsone, a sulfone, are the two most important drugs in this group for the treatment of *P. carinii* pneumonia [8]. Both dapsone and sulfamethoxazole act by inhibiting DHPS and have been found to inhibit the *P. carinii* DHPS [9]. Cotrimoxazole, which is a combination of sulfamethoxazole and trimethoprim (Bactrim, Septra), has been known to be an effective prophylactic and therapeutic agent for *Pneumocystis* pneumonia (PcP) since the 1970s [10,11]. It was officially endorsed as first-line chemoprophylactic agent by the Centers for Disease Control in 1989 [8]. However, trimethoprim appears to have little activity against *P. carinii* in animal models [12,13], suggesting that cotrimoxazole may function as sulfa monotherapy.

Sulfa and sulfone resistance have been well characterized in a variety of bacterial pathogens including *Streptococcus pneumoniae* [14], *Neisseria meningitidis* [15], *Escherichia coli* [16], *Mycobacterium leprae* [17], and *Plasmodium* spp. [18]. Sulfa resistance in these organisms results from point mutations in the DHPS gene. In malaria, mutations tend to accumulate over time and impart greater degrees of drug resistance [19,20].

Since *P. carinii* has been widely exposed to sulfa, it is reasonable to expect that it might be developing sulfa resistance. However, drug resistance in *P. carinii* cannot be confirmed by classical methods because it is not possible to culture patient isolates and determine their in vitro drug sensitivities. Thus, the only way to detect resistance at present is indirectly, by looking for mutations in the *P. carinii* DHPS gene. In 1997 the *P. carinii* DHPS gene was sequenced from six patient isolates, and specific genetic polymorphisms were found [21]. All of the polymorphisms were nonsynonymous (i.e., all resulted in changes in the encoded amino acids). This suggested for the first time that there was evolutionary selective pressure on *P. carinii*, possibly as a result of drug exposure.

Based on homology to the *E. coli* enzyme, whose three-dimensional structure has been solved [22], two of the mutations first found are at the enzyme active site (Fig. 1). Threonine 55 and arginine 56 are involved in binding to the

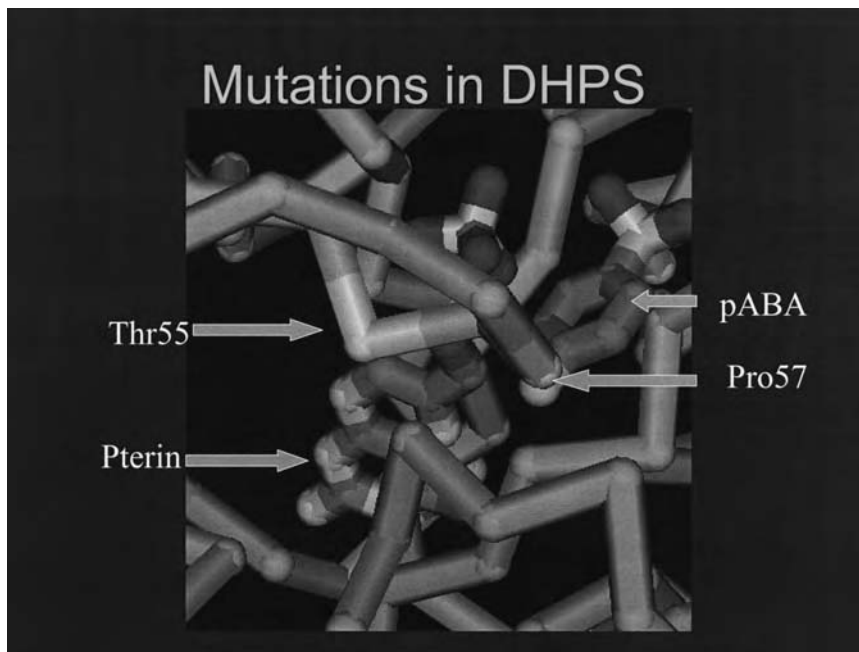


Figure 1 Mutations at the DHPS active site. [Based on the *E. coli* enzyme structure obtained from the NCBI website, <http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=7742> [22].]

pterin substrate; arginine 56 is also involved in binding to pABA and to sulfa. Mutant strains usually replace the threonine at 55 with an alanine, which lacks the hydroxyl group involved in binding. The proline at 57 is also replaced with a serine, which we suspect may alter the position of the critical arginine 56. Mutations at the same sites have recently been shown to cause sulfa resistance in *M. leprae* [17], *Toxoplasma gondii* [23], and *Plasmodium falciparum* [24].

The two mutations at positions 55 and 57 have now been observed in *P. carinii* patient isolates from many other studies. Table 1 summarizes eight published studies in which the frequencies of mutations in sulfa-exposed and nonexposed patients were compared. The studies range in sample size from 17 to 142 patients. DHPS mutations occurred 1.5 to 4.5 times as frequently in patients receiving prophylaxis compared to those not receiving prophylaxis. Thus, patients who are exposed to sulfa drugs are more likely to become infected by mutant strains than those who were not exposed. This suggests that sulfa prophylaxis may provide better protection against wild-type strains than against mutant strains, but this remains to be proven.

Table 1 Association Between Sulfa/Sulfone Prophylaxis and Presence of *P. carinii* DHPS Mutations

Proportion with mutation (%)		<i>p</i> Value	Reference
Sulfa/Sulfone prophylaxis	No sulfa/sulfone prophylaxis		
5/7 (71%)	2/13 (15%)	0.022	27
118/29 (62%)	13/123 (11%)	0.0001	28
11/13 (85%)	3/19 (16%)	0.011	25
5/5 (100%)	3/12 (25%)	0.01	47
28/37 (76%)	14/60 (23%)	0.001	48
57/71 (80%)	19/40 (47.5%)	0.001	49
3/4 (75%)	3/14 (21%)	0.08 ^a	50
6/31 (19%)	3/76 (4%)	0.017	51

^a Calculated using Fisher's exact test.

One other important piece of information was added by Ma and colleagues [25]. Their study was the first to look for mutations in DHFR, the target of trimethoprim. Even though they found many isolates with DHPS mutations, no DHFR mutations were found. This study is consistent with the hypothesis that trimethoprim-sulfamethoxazole may function as sulfa monotherapy.

Do the DHPS mutations arise *de novo*, or are mutant strains spread from person to person? Mutant strains have increased in prevalence over much of the 1990s [26–28]. One study, in fact, showed that the percentage of specimens with DHPS mutations has risen from 0% in 1991 to 55% in 1999 [48]. In addition, the recent observation that mutations may appear in as many as 70% of patients who have never been exposed to sulfa suggest that mutant strains may be spread from person to person [48]. Geographical variations in the prevalence of these mutations in patients with no sulfa exposure, ranging from 13 to 70%, are also consistent with this possibility [26,29]. It is also possible that these differences may reflect the variation in choice of chemoprophylactic agents in different medical practice settings. However, if person-to-person spread of mutant strains occurs, one would expect to see the mutations at higher frequencies in certain strain types. DHPS mutations do not appear to be strongly associated with specific strain types [30,31], but the implications of this finding are uncertain, since the DHPS gene and genotype marker genes might simply not be linked.

III. Clinical Significance of DHPS Mutations

The occurrence of PcP in patients receiving sulfa prophylaxis suggests that the DHPS mutations confer low-level resistance that can overcome the inhibitory

Table 2 Association of Between DHPS Mutations and Response to Cotrimoxazole Therapy

Study site	Study size, WT/mutant	Effects of mutation on		Reference
		Treatment	Survival	
Denmark	115/29	None	↓ 3-months	28
U.S.	21/45	↑ Failure	None	48
Japan	18/6	↑ Failure	—	52
U.S.	36/66	↓ Failure	None	53

effects of the low doses of sulfa administered during prophylaxis. What is less clear is how the mutations affect response to therapeutic doses of trimethoprim-sulfamethoxazole. The results of four studies are summarized in Table 2. The Danish study [28] showed that patients with mutant DHPS were significantly less likely to survive for 3 months than were patients with wild-type *P. carinii* infections; two others did not find this effect. A U.S. study showed an association between mutations and increased treatment failure; although only a minority of patients with DHPS mutations fail sulfa therapy (28%), the failure rate was higher than those with a wild-type strain (10%) [48]. Another showed an increased drug failure rate in patients with mutant *P. carinii*, a third study showed no effect and the fourth showed a decreased failure rate. Thus, it appears that the 55 and 57 mutations in the *P. carinii* DHPS have, at most, a small effect on response to therapy. Nevertheless, it appears that the higher concentrations of sulfa or sulfone in pulmonary tissues achieved with therapeutic doses of dapsone or trimethoprim-sulfamethoxazole result in successful treatment of the majority of patients with PcP who harbor DHPS mutations. But what will happen when a strain with additional mutations arises? If and when that occurs, it could mean a much higher level of resistance to *P. carinii*, as has been witnessed in penicillin resistance in *Streptococcus pneumoniae*. The clinical consequence of this possibility would be loss of the most effective drug for treating PcP.

IV. Atovaquone and Cytochrome b Polymorphisms in *Pneumocystis carinii*

Atovaquone inhibits electron transport in *P. carinii* [32]. The antipneumocystis activity of atovaquone may be enhanced by its effect on a second target – ubiquinone biosynthesis [33]. For malaria, the evidence that atovaquone acts by binding to cytochrome b is overwhelming, and is both biochemical [34], and molecular [35–37]. Furthermore, biochemical and molecular studies on stigmatel-

lin and UHDBT, two electron transport inhibitors which resemble atovaquone, show that they bind to the Q₀ box in bacteria, fungi, and protozoa [38]. Atovaquone's binding to the Q₀ site of the *P. carinii* cytochrome b has been confirmed by three-dimensional modeling (Fig. 2) [39].

While atovaquone was found to be an effective antimalarial, resistance to it developed very rapidly both in vitro [40] and in vivo [41]. To slow down the development of drug resistance, atovaquone is now used for malaria only in combination with another antimalarial drug, proguanil [42]. The rapidity by which resistance to atovaquone develops in malaria is consistent with what is known about its mechanism of action. Cytochrome b, its target, is encoded on the mitochondrial genome, where spontaneous mutation rates are tenfold higher than in the nucleus [43].

The *P. carinii* cytochrome b gene sequences were first reported in 1998 [44]. Mutations were seen in the Q₀ box from two patients on atovaquone prophylaxis. In a later study, cytochrome b genes were sequenced from 60 patient isolates [45]. Of 15 patients with atovaquone exposure, 5 had *P. carinii* cytochrome b mutations, while mutations were seen in only 3 of 45 matched controls with no

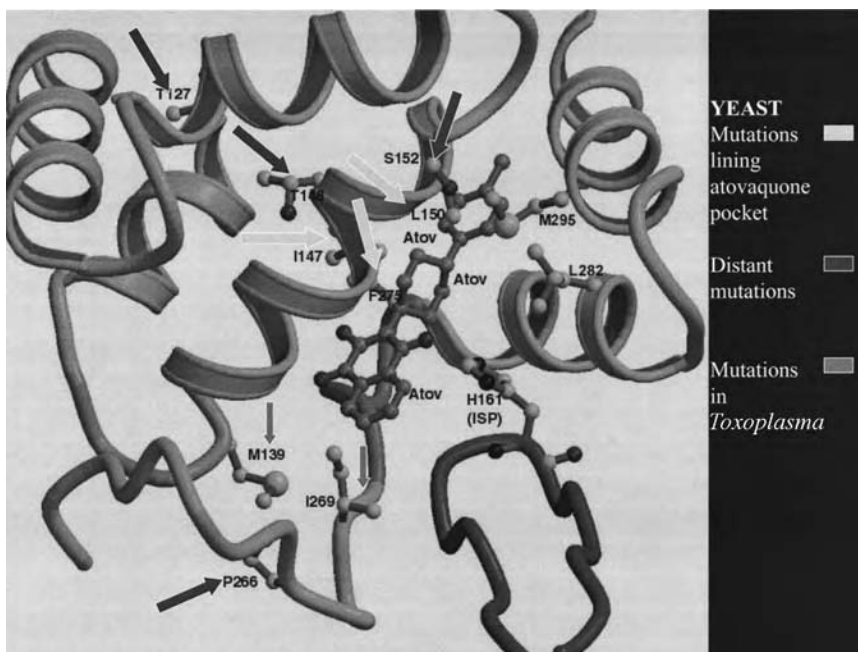


Figure 2 Mutations in the cytochrome b relative to the atovaquone binding site. (From Ref. 39.)

atovaquone exposure ($p = 0.019$). Thus, mutations in the *P. carinii* cytochrome b are significantly more common in patients exposed to atovaquone. In this study, however, the response to atovaquone as therapy could not be assessed, as this agent was not used for this purpose. There were seven different mutations in the Qo box; only two were found in more than one patient. The multiplicity of mutations suggest that atovaquone resistance develops de novo in each patient. Five of the mutated amino acids are in close proximity to the atovaquone binding site, modeled on the yeast enzyme [39].

It has been suggested that other mutations might be responsible for atovaquone failure in the patients who did not have cytochrome b mutations [46]. However, as in most epidemiological studies, the correlation between exposure (mutation) and outcome (drug failure) was not perfect. Reasons for this may include other factors, such as poor compliance and variations in degree of immunosuppression.

V. Summary

P. carinii, like other pathogens, can develop resistance to antimicrobial agents, particularly those used in chemotherapy. Currently, only low-level resistance exists. The possibility that high-level resistance might appear in the future needs to be appreciated.

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Development of Candidate Anti-*Pneumocystis* Drugs: In Vitro and In Vivo Approaches

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I. Introduction

Despite advances in the treatment of the human immunodeficiency virus (HIV) infection, *Pneumocystis jirovecii* pneumonia remains a leading cause of opportunistic infection and mortality in HIV patients. Currently available anti-*Pneumocystis* drugs are limited by significant problems of efficacy, toxicity, and emerging resistance [1–4]. Drug development has been limited by the lack of a reliable and reproducible in vitro cultivation system. In addition, pharmaceutical companies have demonstrated little interest in devoting resources to developing new treatments for *Pneumocystis* and other opportunistic pathogens because of the limited market for these agents. To fill this void, the National Institutes of Health (NIH) established contracts with *Pneumocystis* investigators who have expertise in drug development to discover new anti-*Pneumocystis* compounds and to test agents synthesized by other researchers. The ultimate goal has been to develop new agents or the new use of already licensed drugs for clinical trials for the treatment and prevention of *Pneumocystis* pneumonia.

We have had NIH contracts for *Pneumocystis* drug development since 1984. In the previous (1994) edition of this book, we summarized our experience in evaluating candidate anti-*Pneumocystis* compounds [5]. In the present chapter,

we review the literature for developing anti-*Pneumocystis* drugs and summarize our experience since 1994.

II. Studies in Animal Models

Although *Pneumocystis* organisms obtained from different mammalian hosts are morphologically quite similar, there is now clear evidence supporting the existence of genetic diversity and host specificity within the genus [6–8]. The dramatic genetic diversity among different *Pneumocystis* populations motivated attendees at a recent scientific meeting to begin the process of formally recognizing species [9]. The human form of *Pneumocystis* is now known as *P. jirovecii*, while the most common form in rats is *P. carinii* [10]. Besides the obvious genetic diversity among the species of *Pneumocystis* infecting different mammalian hosts, there is in some cases a distinct level of diversity among populations infecting the same host. To date, rats and ferrets are the only mammalian hosts known to harbor more than a single species of *Pneumocystis* [7,11]. The two known and formally described species of *Pneumocystis* harbored by rats are *P. carinii* and *P. wakefieldiae* (formerly *P. carinii* f. sp. *ratti* [12]. *P. carinii* is the predominant species found in most of the rats from commercial vendors, but these populations can sometimes be found coexisting within the same rat lung [13,14]. This information has had important practical applications. For example, molecular typing techniques have enabled investigators to characterize the species of *Pneumocystis* present in their rat colonies and to begin to evaluate the influences of single vs. mixed populations on the pathology or drug susceptibility of *Pneumocystis* pneumonia [13–16]. Molecular characterization of human *P. jirovecii* isolates has revealed important data about transmission, geographic distribution, and antimicrobial resistance [1,4,17].

A. Rats

Animal models have been the traditional investigative tool for evaluating new compounds for anti-*Pneumocystis* activity. The immunosuppressed rat model of *Pneumocystis* pneumonia developed by Frenkel et al. [18] in the 1960s has served as the basis for most of this work. Rats administered corticosteroids for about 8 weeks “spontaneously” develop *Pneumocystis* pneumonia with histopathological features identical to those of the disease in humans. *Pneumocystis* may have been acquired recently or in the distant past; the mechanism of the development of *Pneumocystis* pneumonia is provocation of low-level infection by chronic administration of immunosuppressive agents to the mammalian host. Systematic use of this model to test new drugs began in the 1970s [19] and has increased over the past two decades. Overall, the rat model has proven to be a reliable predictor of activity in humans; candidate drugs usually have to demonstrate anti-

Pneumocystis activity in this system before the U.S. Food and Drug Administration (FDA) will permit studies in humans.

Investigators have traditionally used adult Sprague-Dawley rats, although a number of other strains (e.g., Lewis rats) work as well. Both male and female rats have proven to be satisfactory for drug testing. For many years, investigators obtained rats from commercial breeders, where they had been raised in a conventional colony and become latently infected with *Pneumocystis* and rodent viruses through natural environmental exposure [20]. As vendors began to develop rat colonies free from viruses, they also appeared to eliminate *Pneumocystis* harbored by these virus-negative rats [21]. However, the picture recently became more complicated, when molecular detection methods showed that commercially supplied rats considered free from *Pneumocystis* were latently infected with the organism [22,23].

The presence of latent *Pneumocystis* infection has not materially affected drug testing but may have significant implications for epidemiological or immunological studies. Investigators have still been able to obtain good levels of *Pneumocystis* pneumonia by immunosuppressing new rats and either housing them with rats with *Pneumocystis* pneumonia ('seeding') or inoculating them intratracheally with live *Pneumocystis* [24,25]. The seeding technique more closely mimics *Pneumocystis* infection in humans, whereas intratracheal inoculation provides control over the inoculum and a somewhat shorter time to reach peak infection. In addition to corticosteroids, *Pneumocystis* pneumonia can be induced in athymic (nude) rats [26,27] and in CD4 cell-depleted rats [28].

B. Mice

The presence of *Pneumocystis* in mice was briefly explored in the 1960s [18], but the first studies establishing immunosuppressed normal mice and congenitally immunodeficient mice as models of *Pneumocystis* pneumonia were reported in the late 1970s [29–31]. The use of the mouse model increased gradually over the next decade [32–38]; in recent years, mice have gained more popularity as models for chemotherapy and immunotherapy of *Pneumocystis* pneumonia [39–47]. Mice offer several advantages over rats: (1) They are only about one-tenth the size and thus consume smaller quantities of drugs, which is very important when the compounds of interest are in short supply; (2) mice have lower housing and maintenance costs; and (3) mice have highly developed genetic and immunological systems that provide a wide variety of strains and reagents for investigational use. The major limitation to mice is that little is known about mouse-derived *Pneumocystis*; most of the molecular tools and immunological reagents have been developed for rat *Pneumocystis*.

Pneumocystis pneumonia can be induced in normal mice by the administration of corticosteroids in a manner similar to that in rats [30,48]. Immunodeficient mouse models of *Pneumocystis* infection have included athymic (nude) mice,

which lack T cells; *uMT* mice, which lack B cells; severe combined immunodeficiency (SCID) mice, which lack B and T cells; and a variety of knockout mice [49]. Some investigators have also administered steroids to speed the development of *Pneumocystis* pneumonia in immunodeficient mice [50]. Another approach in normal mice has been to deplete the animals of CD4 cells, either by administration of a monoclonal antibody (MAb) or the hybridoma itself [46,47]. CD4 cell depletion results in less immunosuppression than that achieved with steroids; this is probably the reason why some drugs exhibit greater anti-*Pneumocystis* activity in mice given anti-CD4 antibody [51]. There is also some question about which immunosuppressive regimen more closely mimics HIV infection. Depletion of CD4 cells resembles one of the prominent clinical manifestations in HIV patients. On the other hand, HIV infects a variety of cell types, and its effects on the immune system are essentially global; this situation is more closely approximated by steroids. While steroids have potential limitations (e.g., drug interactions), these have not proven to be problems in drug testing over years of use.

C. Experimental Protocols

In general, the methods to develop *Pneumocystis* infection and evaluate the efficacy of candidate drugs are similar in rats and mice. Corticosteroids have been the most commonly used type of immunosuppressive agent, although a number of other compounds (e.g., cyclophosphamide, cyclosporine) have also been used [5,23,52]. Administration of a low-(8%) protein diet also enhances the effects of the steroids, but it is quite expensive and used less frequently than in the past.

Since any type of immunocompromised state increases the risks of other microbial and viral infections, barrier facilities with autoclaved food, water, and bedding have been increasingly employed as a means to help control the occurrence of secondary infections. The use of hyperchlorinated drinking water or the addition of an antibiotic to the water is also common. Drug studies last 6 to 12 weeks and are mainly of two types: *treatment*, in which the candidate anti-*Pneumocystis* agents are given over the last 2 to 3 weeks of immunosuppression, and *prophylaxis*, in which the agents are administered throughout the period of immunosuppression. (Studies of relapse of *Pneumocystis* pneumonia have been infrequent.) The rationale for 2 to 3 weeks of time for administration in treatment studies is that most anti-*Pneumocystis* drugs are slow-acting; echinocandins and papulocandins, which can eliminate *Pneumocystis* within a few days, are notable exceptions [53,54]. Drugs have been given by a variety of oral, parenteral, and aerosol routes that can materially affect pharmacokinetics, metabolism, and efficacy. For example, the results achieved by administering a drug by oral gavage may differ from results achieved by mixing the drug in the food or water and allowing the rats to ingest it ad libitum.

Drug efficacy has generally been assessed by analyzing the extent or severity of *Pneumocystis* pneumonia rather than by survival, because most of the

animals live until the end of an experiment; of those that do not survive, it is often not possible to determine whether they died from *Pneumocystis* pneumonia, another microbial or viral infection, or drug toxicity. Severity of infection has been evaluated using a variety of different scoring systems, based either on organism quantitation in lung homogenates, imprint smears, or histopathological staining [5]. Stains to identify *Pneumocystis* have usually been of one of two types: (1) stains such as methenamine silver, toluidine blue, or cresyl echt violet, which selectively stain *Pneumocystis* cysts but not its other developmental forms, or (2) stains such as Wright-Giemsa or one of its more rapid variants (Diff-Quik), which stain the nuclei of all *Pneumocystis* life-cycle stages. In general, counts of nuclei are about tenfold higher than counts of cysts. Immunofluorescence and other immunological techniques have also been used. The lack of standardization among these techniques has deterred direct comparison of the results from different labs.

D. Active Drugs

Drugs that have been tested in experimental models of *Pneumocystis* pneumonia have ranged from agents in clinical use to newly synthesized compounds. Agents that have been effective against *Pneumocystis* in rats have usually been effective in mice, and vice versa. One possible exception was diacetyldapsone, a prodrug that can be metabolized to dapsone in mice but not in rats [55]. This compound was found to be inactive in our rat model of *Pneumocystis* infection. The following classes of drugs have shown anti-*Pneumocystis* activity in rats and/or mice: antifolate compounds, including inhibitors of dihydropteroate synthase (sulfonamides, sulfones, and sulfonylureas) alone or in combination with inhibitors of dihydrofolate reductase (DHFR) [18,19,48,56–70]; sulfonamides combined with macrolides [71–73]; diamidines and related cationionic compounds [74–90]; 8-aminoquinolones alone or combined with clindamycin [91–95]; purine nucleoside analogues [66–95]; polyamine inhibitors [96–101]; nitrofurans [102]; papulocandins and eichinocandins [53,54,103–108]; sodarins [109,110]; allylamines [111–113]; streptogramins [114]; fluoroquinolones [115]; hydroxynaphthoquinones alone or combined with DHFR inhibitors, macrolides, or rifamycins [50,116–119]; ionophores [120]; benzonaphthacenes [121]; iron chelators [122–124]; and immunological agents (e.g., cytokines, lymphocytes, antibodies, vaccines) [125–134] as well as compounds with novel mechanisms of action [135]. Several reports have examined the efficacy of drug combinations in animals infected with *Pneumocystis* and other microbial pathogens [136–142].

III. In Vitro Systems and Drug Screening

During the course of our contract work, we conducted extensive *Pneumocystis* culture, life-cycle, and drug screening studies using monolayers of the A549 cell

line and a transformed lung fibroblast line [143–147]. A standard method of *Pneumocystis* enumeration by counting of Giemsa/Diff-Quik stained organism nuclei rather than a selected life-cycle stage (e.g., cyst, trophic form) and use of the rapid variant of the Giemsa stain, Diff-Quik, for this purpose was established [145,146]. The rapid Giemsa stain has been adopted by almost every laboratory employing microscopic enumeration of *Pneumocystis* nuclei (sometimes referred to as “trophozoites”) as well as in the clinical laboratory for rapid diagnosis of *Pneumocystis* pneumonia [148]. *Pneumocystis* was counted in preparations prior to inoculation to avoid a false impression of growth due to release of the organisms from the monolayer over time. Surprisingly, other systems used counts on day 1 as the starting point for a study. Our observations and experience with these tissue culture systems made it apparent that use of monolayers for in vitro drug screening was problematic. Unless a viability stain was included in the counts over the culture period, dead *Pneumocystis* could stain with Diff-Quik or a cyst stain and lead to misinterpretation of a drug’s effects. In fact, we found that organisms could be stained with Diff-Quik after 4 weeks in culture. Our later studies using quantification of ATP as a viability assessment of the *Pneumocystis* organisms verified the lack of viability in cultures where the *Pneumocystis* stained with Diff-Quik [149,150].

Certain drugs could influence the viability and metabolic processes of the monolayer cells, leading to an incorrect conclusion of efficacy for *Pneumocystis*. For example, treatment of A549 monolayers with the protein synthesis inhibitor muconomycin A, followed by extensive washing and inoculation of untreated *Pneumocystis*, inhibited *Pneumocystis* replication. Not all inocula produced the same level of replication in vitro, leading us to exclude all experiments where untreated *Pneumocystis* failed to increase threefold. Besides these concerns, we found it difficult to evaluate uptake of radiolabeled precursors without interference by the monolayer, although attempts were made to block metabolism radio-metrically or by chemical means (e.g., gamma radiation, muconomycin A).

Because of the inherent problems with tissue culture, we explored the use of cell-free systems for growth and drug screening of *Pneumocystis* [151]. Using a peptone-based medium, we observed an increased incorporation of [³⁵S]-methionine into *Pneumocystis*-specific proteins over time, which did not occur in *Pneumocystis* maintained in Dulbecco’s minimal essential medium (DMEM). There was a concurrent modest increase in *Pneumocystis* nuclei per milliliter (five- to sevenfold), which suggested that the organisms could maintain viability in a cell-free system. The *Pneumocystis* organisms were responsive to the standard anti-*Pneumocystis* compounds trimethoprim-sulfomethoxazole (TMP-SMX) and pentamidine in that they did not increase over inocula levels. Viability assay using erythrosin-B dye exclusion showed many of the organisms were dead, yet they still stained with the Diff-Quik stain. Thus, the same phenomenon that occurs in monolayer-based cultures (i.e., dead *Pneumocystis* stained with dyes used for

enumeration) also occurs in this cell-free system. We feel this is a major shortcoming of in vitro screens solely based on counting of *Pneumocystis*.

These problems led us to explore the use of ATP content, a global assessment of viability, to screen drugs [149,150,152–154]. ATP is a universal energy unit in almost all living cells. When a cell dies, ATP is rapidly degraded by ATPases; the decline in ATP levels permits an immediate assessment of cell viability. ATP reflects the viability of an entire cell and does not target a single enzyme or pathway. The luciferin/luciferase driven bioluminescent ATP cytotoxicity assay has been shown to be a simple, rapid, and sensitive test to measure ATP levels in a variety of eukaryotic and prokaryotic cell systems [155–157]. We have found that the response of *Pneumocystis* to test compounds in the ATP assay could be assessed within hours rather than over a period of days by microscopic enumeration [150]. The assay also enabled us to discern any discrepancies between microscopic counts of the *Pneumocystis* and viability of the *Pneumocystis* populations. The use of multiwell plates reduced reaction volumes and the need for large numbers of *Pneumocystis* and amounts of drugs while decreasing the time needed for setup, sampling, and analysis. Significantly, the measurement of evolved light by the luminometer provided an unbiased quantitation of a drug response (ATP levels) in contrast to enumeration of organism numbers that could be influenced by technician bias or error. Our experience indicates that the ATP assay is a highly efficient system of screening candidate drugs. The ATP levels also correlated well with data obtained in our rat model: the ATP assay had a true positivity rate of 73%, a true negative rate of 89%, a specificity of 84%, and a sensitivity of 80% [150]. These values were comparable with other in vitro assays of drug screening (e.g., the Ames test for mutagenic potential) and animal studies. The ATP assay also correlates well with results obtained in our mouse model of *Pneumocystis* pneumonia.

We have also adapted the ATP assay system to assess the potential toxicity of promising candidate compounds identified in the *Pneumocystis* in vitro system to mammalian cell monolayers (i.e., A549 lung cells) as an intermediate screening step prior to testing in the animal model of *Pneumocystis* pneumonia [114]. We feel this step is an important one that has helped to screen out compounds that may produce toxicity in the animals and for guidance in determining the dosage in the animal model. Potentially toxic compounds were routinely started at lower dose-finding ranges than apparently nontoxic ones, for example.

IV. Recent Studies

In this section we summarize most of the drug studies performed in our laboratories since the last edition of this book. A large portion of this work involved the development, validation, and application of the ATP assay to screen candidate drugs. Unfortunately, a large number of compounds (about 150) have not been

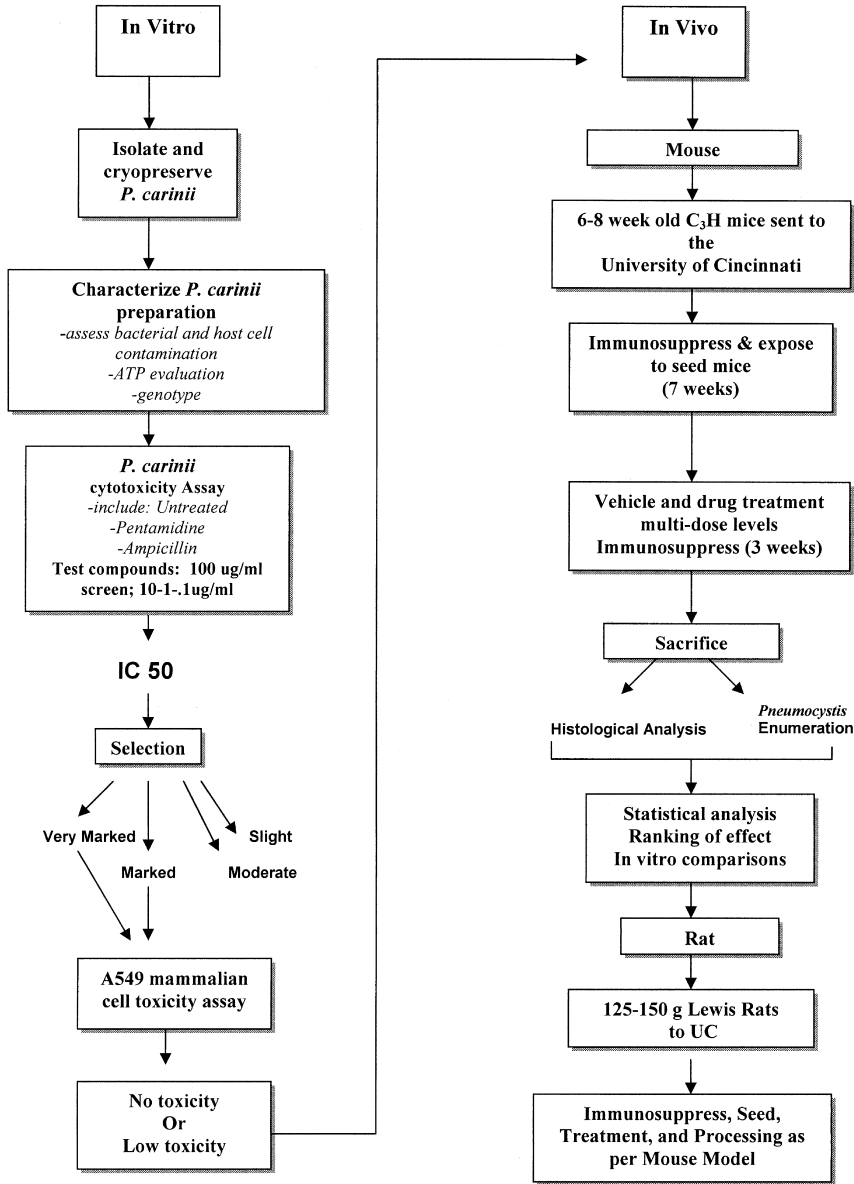


Figure 1 Schematic for selection of candidate anti – *Pneumocystis* compounds.

included because of pending patent applications or the proprietary nature of the compounds. Our scheme for drug screening is presented in Figure 1.

A. In Vitro Studies

Source and Preservation of Pneumocystis

Pneumocystis used in our in vitro studies is obtained from immunosuppressed rats with *Pneumocystis* pneumonia housed in the following manner: (1) Long Evans and Brown Norway rats bred and maintained under conventional conditions at the Cincinnati Veterans Affairs Medical Center (VAMC) and (2) CD rats obtained from Charles River Laboratories (Hollister, CA) maintained under barrier conditions at the University of Cincinnati (UC) and inoculated intratracheally with *Pneumocystis*. Lungs from animals with fulminant *Pneumocystis* infection are excised with a minimum of blood and host cells; minced with scissors, and homogenized with a Stomacher® lab blender; the resulting supernatant is sieved through sterile gauze and centrifuged at high speed (i.e., $(1000 \times g)$, which collects both organisms and host cells. *Pneumocystis* preparations are subsequently treated with aqueous ammonium chloride (0.85%) to lyse host red blood cells and subjected to low-speed centrifugation to separate the larger host cells from the organisms. After reconstitution in a buffered saline solution, the preparations are filtered twice through 10 μm filters to further reduce host cell contamination. Purified *Pneumocystis* are enumerated; cryopreserved in an RPMI-1640-based medium containing 10% fetal calf serum and 7.5% DMSO, and stored in liquid nitrogen. A single infected rat lung can yield up to 2×10^{10} organism nuclei; the vast majority (about 95%) of the life-cycle stages in these preparations are trophic forms, with the remainder (about 5%) being composed of cysts. *Pneumocystis* organisms from each rat lung are genetically characterized by CHEF analysis. Approximately 25% of a *Pneumocystis* homogenate is prepared for CHEF analysis by treatment with DNase, washes in EDTA, embedment in 0.8% agarose, and proteinase K-SDS lysis with subsequent storage at 4°C in 0.5 M EDTA [13]. CHEF conditions are described elsewhere [13,16]. The predominant form of *Pneumocystis* used in our studies was *P. carinii* form 1; in some cases, a mixed infection of *P. carinii* and *P. wakefieldiae* was present [7,12,15].

Each batch of processed *Pneumocystis* is evaluated for microbial contamination, ATP content, karyotype, and host cell content prior to use in the ATP assay. After cryopreservation (described above) and enumeration, the test batch of *Pneumocystis* organisms is thawed and cultured in RPMI medium for 3 days. Samples are first checked for obvious microbial and host contamination by phase-contrast microscopy and after staining with Gram's stain and Diff-Quik. If there is no fungal or bacterial contamination and if the host cell numbers are low, the organism preparation is then checked for ATP content. Once cleared, the karyotypes of the preparation are established by CHEF analysis and, at times, by poly-

merase chain reaction (PCR) targeting the mitochondrial large subunit rRNA (mtLSU) [158]. The mtLSU is used only to detect the presence of cryptic mixed *Pneumocystis* infections [159] or to determine the stability of the genome. When genotyping and ATP analysis are complete and satisfactory, the organisms are cleared for experimental use. These procedures have not only improved the reliability and reproducibility of the ATP assay but also enabled us to reduce the overall number of immunosuppressed animals used for in vitro studies [154].

ATP Assay

The basic principle of the bioluminescent reaction relies on the quantitative measurement of light produced as a result of an enzymatic reaction between the substrate, luciferin, and the enzyme, luciferase, catalyzed by ATP. The amount of light generated by the reaction is linearly related to the number of molecules of ATP present in the system:



In the first step (1), D-luciferin (LH₂) and ATP in the presence of luciferase (E) react to form luciferyl adenylate (LH₂-AMP), which remains tightly bound on the catalytic site of luciferase (E), with subsequent liberation of inorganic phosphate;



Upon exposure to molecular oxygen, the enzyme-bound luciferyl adenylate is oxidized to yield oxyluciferin (P), which emits light upon returning to ground state. Carbon dioxide and AMP are produced. One quantum of light (L) is emitted for each molecule of luciferin oxidized. The oxyluciferin remains bound to the luciferase and cannot regenerate and enter into the reaction again.

“Batch qualified” (passing the process described above), cryopreserved organisms are thawed rapidly; centrifuged to remove the cryoprotectants and resuspended in RPMI medium with 20% calf serum and other additives (e.g., nonessential amino acids); at pH 7.5 to 8; and 380 mOsm [149,150]. Drugs to be tested are prepared in culture medium or, if necessary, in DMSO or ethanol (final concentration <0.2% vol/vol) and 5×10^7 nuclei/mL are added in 0.5 mL of culture medium to each well of a 48-well plate. Each drug concentration is studied in triplicate. Media without drug and with 10 µg/mL ampicillin serve as the negative controls. A set of triplicate wells receives pentamidine isethionate at 1 µg/mL (the 48-hr IC₅₀) as the positive drug-activity control. The plates are incubated at 35°C, 5% CO₂. At 24, 48, and 72 hr, the wells are agitated, and 10-µL samples from each well are removed and placed directly into individual wells of a 96-well opaque white plate containing 3.5% trichloroacetic acid in 200 µM TRIS (pH 1.5) to release intracellular ATP; they are then stored at -20°C (final volume 40 µL). The culture

plates are centrifuged to sediment the *Pneumocystis* on the bottom of the wells to facilitate removal of 250 μ L of supernatant. An equal amount of fresh medium with drug/no drug is then added back to each well. We found that this feeding step facilitates stabilized nutrient levels and pH, resulting in increased ATP levels over time in untreated controls. Each plate is loaded into a FluoSTAR Optima Plate Reader (BMG Labtechnologies, Inc.) for assessment of ATP. The samples are automatically mixed with the luciferin/luciferase reagent via an injector and immediately measured for light emission at 562 nm. Each well is read in triplicate and expressed as the average relative light units (RLUs).

In addition to the negative and positive controls for drug activity mentioned above, a quench control is run for every drug tested. This control evaluates whether the drug itself may have an inhibitory effect on the enzyme-substrate reaction. The highest concentration of a drug used in an assay is directly placed in triplicate wells containing 10^{-8} M ATP, luciferin, and luciferase. Partner wells receives no drug, but the reaction reagents are included. Both sets of wells are read for light evolution, which should be identical. Thus far, the only compound we have observed to interfere with the reaction was sodium azide.

Each compound is initially evaluated at 100 μ g/mL to screen out inactive compounds. If a 50% decrease in ATP compared to untreated organisms is observed for the compound, a three-concentration series is then run to determine the IC_{50} (10, 1, and 0.1 μ g/mL). Compounds with a high degree of activity (reduction to less than 50% of control at all concentrations) are tested again at nanogram levels.

The effects of drugs on the ATP of *Pneumocystis* pools are calculated by the following formula:

$$A - B/A \times 100 = \% \text{ decrease in ATP}$$

where A = the average Relative Light Units (RLU) of the untreated and ampicillin treated wells from which the Background RLU (B-RLU) has been subtracted and B = the average RLU of the drug-treated wells from which the B-RLU has been subtracted

Data Analysis: IC_{50} Rank Scale

The primary method we use for evaluating the effects of drugs on *Pneumocystis* in vitro is based on the concentration of compound necessary to decrease the ATP by 50% of the control *Pneumocystis* ATP at that time point (IC_{50}). The resultant concentration is ranked according to a scale based on in vitro response vs. in vivo response (see below). Those drugs that rank as “very marked” and “marked” are selected for further analysis by host cell toxicity testing, then in vivo studies (see Fig. 1). Recently, we have been using the “moderately effective” drugs in combination studies as an effort to increase their efficacy. We will consider those with “slight” activity for combination studies if their target complements in some way the target of the other drug partner.

The percent decrease in ATP content of a compound is used in a linear regression formula with the log drug concentrations to determine the 50% inhibitory concentration (IC_{50}) (GraphPad Software v2 for Science, San Diego, CA). Based on its IC_{50} value, each agent is classified using an activity scale. The original scale had four categories: very marked, marked, moderate, and none. As our experience with drug testing has increased, we have added a fifth category of activity: slight. This was done in order to have as many categories as our in vivo activity scale and to accommodate drugs that attain serum levels higher than 10 $\mu\text{g/mL}$. The categories in the current drug activity scale are very marked, $<0.1 \mu\text{g/mL}$; marked, 0.1 to 0.9 $\mu\text{g/mL}$; moderate, 1.0 to 9.9 $\mu\text{g/mL}$; slight, 10.0 to 49.9 $\mu\text{g/mL}$; none (inactive), $>50 \mu\text{g/mL}$.

Conditions, Controls, and Applications of the ATP Assay

Host Cell Contamination

One potential problem in interpreting the results of the ATP assay is the potential influence that host cells may have on the overall ATP levels of a given *Pneumocystis* batch. Although we take great care to reduce and/or eliminate host cells from the *Pneumocystis* preparations, we recognize that all such methods are imperfect in their ability to always eliminate all host cell contaminants. In previous studies, we reported a greater than 1000-fold reduction in host cells using the purification method described above [149]. In a more recent study, the viability of host cells as well as the ATP content of the organism pool in four separate *Pneumocystis* preparations was tracked over a 72-hr period. Viability of host cells was determined using the calcein AM/ethidium homodimer staining method [154]. While the ATP content measured as RLUs of all four pools increased from 0 to 72 hr. the numbers of live host cells in the same pools decreased to or below the level of microscopic detection (Table 1).

Additional data supporting the lack of participation by host cell ATP in the assay are the responses of the cultures to specific inhibitory compounds; mammalian cells are quite sensitive to oligomycin B, yet this compound had little to no effect on the ATP levels of the *Pneumocystis* organisms [149,150]. Likewise, TMP-SMX and pentamidine are well tolerated by mammalian cells yet were very effective in decreasing the ATP levels in the *Pneumocystis* preparations.

The ATP Assay

This assay is an exquisitely sensitive indicator of cell viability, enabling a comparison of ATP levels with numbers of *Pneumocystis* nuclei. The following experiment illustrates the problems in attempting to evaluate anti-*Pneumocystis* drugs by using enumeration of organisms as the only method to determine drug efficacy. (Table 2). ATP levels of *Pneumocystis* organisms that had been stored in liquid nitrogen for 26 months remained stable throughout the assay period of 7 days,

Table 1 ATP Levels and Live Host Cells over Time

Donor	0 hr	24 hr	48 hr	72 hr
ATP pools				
01-06#01	8,401	23,671	19,748	24,863
01-06#02	9,952	32,498	10,077	32,081
01-06#11	12,023	31,554	28,660	34,580
01-07#02	12,569	38,004	27,615	27,661
Live host cells/mL				
01-06#01	4.2×10^4	0 ^a	2.1×10^4	0
01-06#02	6.3×10^4	0	2.1×10^4	0
01-06#11	2.1×10^4	4.2×10^4	0	0
01-07#02	1.0×10^5	0	2.1×10^4	2.1×10^4

^a Limit of detection = 2.1×10^4 .

with a characteristic increase from inoculation to 24 hr and a modest decline beginning at day 4 of culture. While counts of nuclei and RLUs followed the same pattern in the untreated control group, measurements of the pentamidine-treated group showed that counts of nuclei lagged behind decreases in ATP levels from 22 to 70% at all time points. These results suggest that intact but metabolically inactive nuclei can persist in the culture medium for several days and produce misleading results in evaluation of drug efficacy.

Short- and Long-Term Analysis of ATP Levels

The rapidity and sensitivity of ATP measurement for determining responses to drugs are demonstrated in the following assay, where the responses of cryopre-

Table 2 Changes in ATP Levels and *Pneumocystis* Nuclei Counts over Time

	Media control		Pentamidine 1 μ g/mL	
	Nucleus counts	RLUs ^a	Nucleus counts	RLUs
Day 0	4.84×10^7	35,847	4.84×10^7	26,553
Day 1	9.36×10^7	79,353	7.45×10^7	22,043
Day 2	9.43×10^7	85,508	6.85×10^7	3,205
Day 3	9.93×10^7	79,336	5.70×10^7	1,643
Day 4	7.20×10^7	80,118	2.85×10^7	1,933
Day 6	5.56×10^7	64,892	3.72×10^7	1,598
Day 7	6.53×10^7	44,926	1.40×10^7	1,153

^a Relative light units.

Table 3 Short- and Long-Term Effects on ATP Levels by Anti-*Pneumocystis* Drugs

Hours	0	1	2	3	4	5	6	24	48	72
Media	9,234	12,837	15,096	18,658	21,074	22,611	24,903	31,205	26,436	52,445
Ampicillin 10 µg/mL		11,738	13,632	18,029	19,845	23,099	23,760	28,265	28,380	67,480
Pentamidine 2 µg/mL		9,477 (19.4) ^a	9,411 (31.2)	11,872 (34.3)	12,221 (38.6)	11,053 (52.4)	9,596 (59.8)	851 (97.3)	270 (99.4)	272 (99.8)
Atovaquone 5 µg/mL		5,490 (53.6)	5,713 (58.5)	4,955 (72.9)	4,245 (78.9)	3,531 (85.0)	3,076 (87.4)	466 (98.7)	256 (99.4)	174 (100)

^a Data in parentheses indicate percent reduction in relative light units per media control.

served *Pneumocystis* organisms to atovaquone and pentamidine were measured at 10 time points after introduction into culture medium (Table 3). During the first 6 hr of incubation, the ATP values in untreated wells (“Media”) increased almost threefold, from 9234 to 24,903 RLUs, while the increase was more modest from 24 to 72 hr (1.7-fold). Pentamidine (2 µg/mL) and atovaquone (5 µg/mL) decreased the ATP values of the *Pneumocystis* pools by 59 and 87%, respectively, during the first 6 hr of incubation. Reductions in ATP for both compounds continued through the 72 hr assay period.

These data highlight our contention that in vitro studies relying on enumeration as an indication of compound cytotoxicity are potentially flawed. Manual counting of cysts or trophs is less sensitive and gives no indication of the metabolic state of the organism. It is more labor-intensive, prone to subjective technical error, and the assay period itself is over twice the length of the ATP assay.

Use of the ATP Assay to Analyze *Pneumocystis* Growth in a Continuous Axenic Culture

Improving the viability of *Pneumocystis* organisms in culture, increasing the replication levels, and maintaining the organisms in vitro for longer periods of time are major concerns of our research group. We continually screen different additives, nutrients, and environmental conditions in an attempt to improve the present system. The results of many trials have led to small advances and changes of our standard culture protocol. For example, we screened different sources of sera (including horse) and found that calf serum provided the same or slightly better results achieved with the more expensive fetal calf serum. We reduced the complexity of our original medium [181,182], so that the only additives besides calf serum are nonessential amino acids, glutamine, and vitamins. In addition, we verified the optimal pH of 8.0 to be the same as in our earlier studies but found better results with a slightly decreased temperature (34 to 35°C) and carbon dioxide percentage (5%). Significantly, we found that any concentration of *Pneu-*

mocystis greater than $5 \times 10^7/\text{mL}$ greatly decreased the ATP levels over time in culture. A variety of fungal media used for different applications (e.g., mating induction) consistently produced dramatic decreases in *Pneumocystis* ATP levels almost immediately upon contact.

Thus, a recent report of long-term axenic cultivation of *Pneumocystis* [160] was greeted with enthusiasm by our laboratory. The axenic cultivation protocol required collagen-coated transwells, twice-daily feeding, and medium containing s-adenosyl-l-methioine (AdoMet), which had to be added twice a day. Four long-term trials of the method led to disappointing results in our hands. Enumeration of nuclei from the cultures was problematic due to low cell numbers and background staining of media components. Washing or dilution of the samples did not improve detection. Staining with Diff-Quik did showed the persistence of some of the inoculated *Pneumocystis* organisms and the presence of enucleate blue-colored entities in some wells. These did not increase over time.

In one experiment, *Pneumocystis* was inoculated into transwells and treated according to the cultivation protocol for 56 days (Table 4). Samples were checked weekly for ATP content and counts of nuclei were made using Diff-Quik stained slides. No organism growth or increase in metabolism was detected.

Components of the axenic medium were tested in our standard culture conditions (Table 5). Experimental groups included our standard RPMI 1640-based *Pneumocystis* culture medium, medium plus AdoMet, medium with horse serum substituted for our usual bovine calf serum, and medium with horse serum and AdoMet. The results indicated that while horse serum was an adequate substitute for calf serum, AdoMet at the suggested concentration was moderately toxic to *Pneumocystis*.

Another laboratory also reported their inability to replicate this culture method [161]. A rapid quantitative touchdown (QTD) PCR assay based on the

Table 4 Adomet-Based Culture System for *Pneumocystis*

Time point	Nucleus count	ATP Content
Day 0	1.0×10^7	1337 RLUs ^a
Day 7	8.8×10^6	Background
Day 14	4.9×10^6	Background
Day 21	1.0×10^7	Background
Day 28	3.8×10^6	Background
Day 35	3.5×10^6	Background
Day 42	4.2×10^6	Background
Day 49	2.8×10^6	Background
Day 56	1.0×10^6	Background

^a Relative light units.

Table 5 AdoMet and Horse Serum as Additives in Different Media

Day	1	2	3	4	5	7
Media	20,524	26,728	37,684	48,957		
AdoMet 500 μ M/mL	26,630	29,538	4,161	174	139	114
20% horse serum	18,808	22,197	26,966	33,616	29,817	37,740
Horse serum + AdoMet	18,606	21,094	17,051	11,382	576	256

single-copy dihydrofolate reductase gene was used to detect *P. carinii*. The technique was highly sensitive and could detect <5 copies of a plasmid standard per tube and was reproducibly quantitative ($r = 0.99$) over six log values for standards containing ≥ 5 copies per/tube. Application of the PCR method to the in vitro axenic cultivation system for *P. carinii* confirmed the microscopic findings that no organism multiplication had occurred during culture [160]. Moreover, all such cultures showed a decrease in *P. carinii* DNA that exceeded the expected decrease due to dilution of the inoculum upon transfer.

Based on our findings and this recent report, we have discontinued tests of this cultivation protocol, having concluded that it is not a robust system and thus not suitable for drug screening, which requires adequate numbers of organisms that can readily be counted and tested for viability. This culture method may find a use in small-scale studies where the intensity of labor required and high cost of medium additives are not issues.

Drugs Analyzed

Sterol Inhibitors

We evaluated compounds targeting enzymes in the sterol biosynthetic pathway in collaboration with Dr. Edna Kaneshiro (University of Cincinnati)[162]. Biochemical analyses have determined that *Pneumocystis* does not contain the common fungal sterol ergosterol as its bulk sterol [163–166] and probably uses cholesterol for this function; however, other biochemical studies have indicated that several intermediates of cholesterol biosynthesis were present in organisms isolated from rat lungs [166]. Studies using sterol inhibitors provide further support of at least a partially operational sterol biosynthetic pathway. Terbinafine, an inhibitor of squalene epoxidase, was shown to be effective against *Pneumocystis* infections in vivo and to inhibit organism growth in vitro [111,112,167]. Likewise, Urbina et al. could inhibit sterol C-24 alkylation in vitro using targeted inhibitors and subsequently decrease proliferation of the organisms in short-term culture as well as inhibiting the biosynthesis of its sterols [168]. Thus, we decided to further explore the biosynthesis of sterols as a potential drug target for anti-*Pneumocystis*

therapy. Compounds inhibiting the various steps in sterol biosynthesis were obtained from commercial sources; as proprietary experimental drugs from Glaxo-Wellcome, Merck, and Ayerst; or were synthesized by E.J. Parish, a medicinal chemist. The enzymatic reactions in the sterol biosynthetic pathway that were the putative targets for these drugs are shown in Figure 2: HMG-CoA reductase ("statins"); squalene synthase, the first dedicated step in sterol biosynthesis

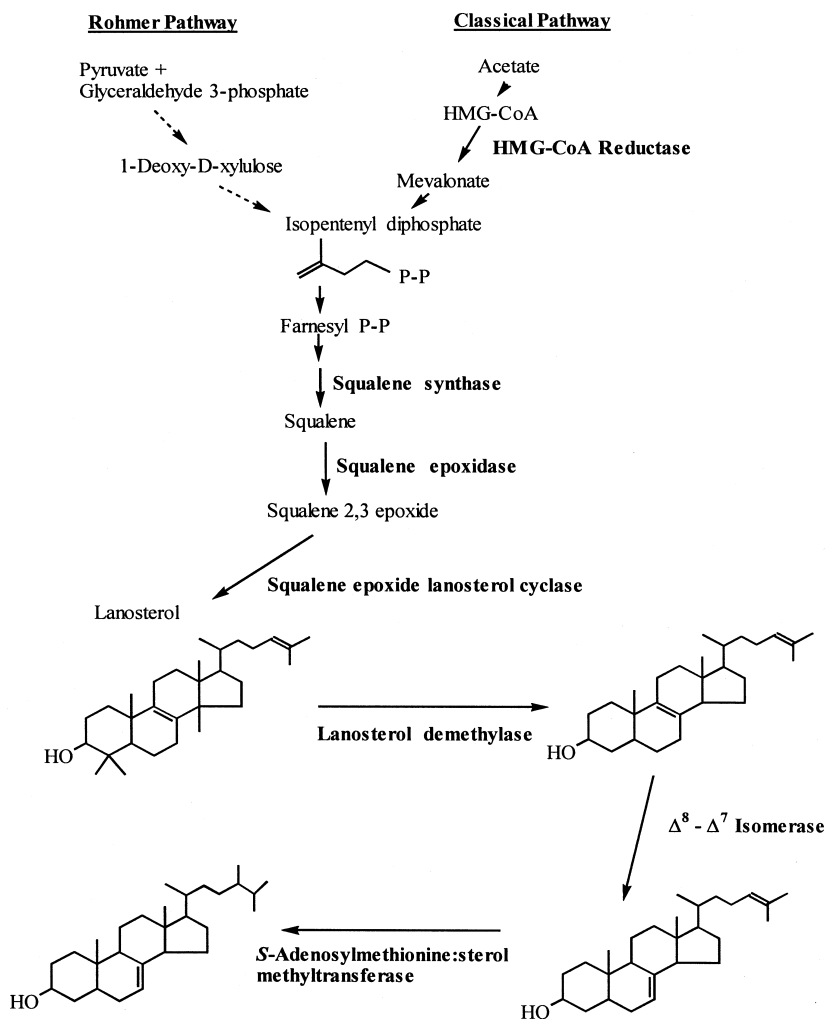


Figure 2 Proposed sterol biosynthetic pathway in *Pneumocystis*. Targets of inhibitors used to probe the synthetic pathway are shown in bold type. (Modified from Ref. 162.)

(squalestatin); squalene epoxidase (terbinafine, tolnaftate); squalene epoxide-lanosterol cyclase (GR compounds); lanosterol demethylase (the “-azoles”); isomerase (AY 9944); and the SAM-sterol methyltransferases (synthesized substrates—e.g., epiminolanosterol).

Results from these studies (Table 6) showed moderate activity for certain compounds inhibiting squalene synthase, squalene epoxidase, squalene epoxide-lanosterol cyclase, and isomerase. Three of the commercially available demethyl-

Table 6 IC₅₀s for Inhibitors at Three Exposure Times as Determined by the Reduction in Cellular ATP Content of *P. carinii* f. sp. *carinii* Populations

Drug	IC ₅₀ (μ mL [μM]) at the following treatment time (hr):		
	24	48	72
HMG-CoA reductase inhibitors			
Simvastatin	— ^a	—	101.39 (2422)
Lovastatin	—	—	—
L-647,318	—	—	—
L-654,164	—	—	—
Squalene synthase inhibitors			
CCI 14993	1244.51 (2882)	7.89 (18)	3.91 (8)
CCI 16543	138.68 (434)	21.83 (68)	10.45 (33)
Squalestatin (GR 105155X)	—	—	—
Squalene epoxidase inhibitors			
Terbinafine	1706.08 (5863)	36.98 (130)	3.67 (13)
Tolnaftate	—	1221.80 (3980)	49.90 (1625)
Squalene epoxide-lanosterol cyclase inhibitors			
GR 90525A	1.89 (7)	1.06 (4)	0.93 (3)
GR 193018A	8.07 (30)	2.82 (11)	2.23 (8)
GR 54985A	40.27 (121)	5.44 (16)	2.85 (9)
UI 8666A	30.76 (212)	8.79 (60)	9.71 (67)
GR 31149A	1419.10 (4464)	14.80 (47)	1.90 (6)
Lanosterol demethylase inhibitors			
GR 40317A	12.33 (33)	2.90 (8)	2.02 (5)
GR 42539X	23.71 (81)	2.33 (8)	1.98 (7)
GR 40665X	194.53 (692)	127.35 (453)	104.47 (372)
Fluconazole	—	—	—
GR 71539X	—	—	—
GR 77303X	—	—	—
Δ ^S to Δ ⁷ isomerase inhibitor, AY 9944	25.06 (51)	17.82 (36)	3.68 (7)
SAM:SMT inhibitors			
24(25)-Epiminolanosterol	—	2,084.49 (4737)	—
24-Bromolanosterol	—	—	—
24-Iodolanosterol	—	—	—
Sinefungin	—	—	—
Polyene antimycotic, amphotericin B	1.84 (2)	1.06 (1)	0.94 (1)

^a Inhibition was always less than 50%.

Source: Ref. 162.

ase inhibitors (e.g., fluconazole) had no effect on the ATP of the *Pneumocystis* pools, but three of the experimental demethylase inhibitors showed moderate efficacy. These results indicate that *Pneumocystis* does have a lanosterol demethylase, but its structure may be distinct from that of other fungal demethylases. Such information offers hope for the development of *Pneumocystis*-specific demethylase inhibitors, which are such an important target in fungi. Coincident with these inhibition studies, several orthologues of the genes in this pathway were identified in gene databases generated by the *Pneumocystis* Genome Project (M. Cushion, Director; <http://pneumocystis.uc.edu>). Significantly, the inhibitory functions probed by the ATP assay could be associated with the presence of the gene in the *Pneumocystis* genome. Thus, the enzymatic steps driven by *erg1* (squalene epoxidase), *erg7* (oxidosqualene-lanosterol cyclase), and *erg6* (SAM-SMT) showed inhibition upon addition of the inhibitory compounds. The lanosterol demethylase gene of *P. carinii* was recently sequenced [169]; its presence could explain the inhibition shown by treatment with the inhibitors GR 40317A, GR 42539X, and GR 40665X (Table 6). The precise structure of the *P. carinii erg11* gene homologue awaits further study, but such structural information should reveal the specific properties that render it refractory to standard azoles. Orthologues to HMG-CoA reductase genes have not yet been detected in the *P. carinii* genome. In the study presented in Table 6, the compounds targeting this step were ineffective at reducing the ATP pools of the *P. carinii* as well. Yet in recent studies performed in our laboratory, other HMG-CoA reductase inhibitors (e.g. simvastatin) were found to be highly active in reducing the ATP of *P. carinii* in culture, (unpublished data). These data suggest that *P. carinii* does have HMG-CoA reductase activity, although the genes have not yet been identified. This can be explained by the fact that the genome has not yet been completed or that the *P. carinii* HMG-CoA reductase genes may have low homology to those fungal genes present in GenBank. Moreover, like the lanosterol demethylase enzyme, these proteins may be sufficiently different from the fungal enzymes to explain the differences in susceptibility to these inhibitors. Although most of these sterol inhibitors exhibited only moderate effects on organism viability, it is our hypothesis that treatment with combinations of these compounds that target different steps in the sterol biosynthetic pathway or other biochemical pathways offer alternative treatment strategies.

Comparative Analyses

In certain cases we use one-way analysis of variance with a multiple-comparisons post test to evaluate the significance of the average RLUs of test compounds vs. control. Since there are usually several groups to be evaluated, a Student Newman-Keuls post test or a corrected *t*-analysis like Bonferroni is used. The α -value is set at 0.05; a *p* value of <0.05 is considered significant. Such an application

occurred when we were assessing the efficacy of different concentrations of atovaquone at early time points (1 to 6 hr) [182] (Fig. 3).

In this experiment, the average $\pm$ SEM RLUs for each drug concentration and control at hourly time points were plotted as histograms and analyzed with the Student Newman-Keuls post test. In the medium control, the RLUs at each hourly increment increased significantly from 1 to 5 hr ($p < 0.05$). In contrast, ATP levels of *Pneumocystis* treated with 10 μg of atovaquone per milliliter had decreased by 70% after 1 hr of exposure and continued to decrease throughout the next 5 hr. Treatment with 5 $\mu\text{g}/\text{mL}$ initially decreased the ATP levels by 40%, with a more gradual decline over 6 hr than was observed for the *Pneumocystis* treated with 10 $\mu\text{g}/\text{mL}$. At all time points, there were significant differences between the control group and those treated with 5 and 10 μg of atovaquone per milliliter ($p < 0.001$). Exposure to atovaquone at 1.0 and 0.5 $\mu\text{g}/\text{mL}$ for 4 hr caused no significant decreases in the ATP contents of the populations. At 5 and 6 hr of exposure, slight but significant decreases were observed at these concentrations ($p < 0.001$). These studies showed a dose-dependent effect of atovaquone on the ATP pools of *Pneumocystis* and support the mechanism of action of this drug to be the organisms' electron transport chain and oxidative phosphorylation.

Use of the ATP Assay to Evaluate Drug Toxicity to Mammalian Cells

Compounds demonstrating marked or very marked activity on the rating scale are subsequently tested for toxicity to mammalian cells before introduction into the mouse *Pneumocystis* pneumonia model. The ATP assay has been adapted for evaluating the viability of monolayer cells in much the same way as for *Pneumocystis*. The IC_{50} of a test compound is calculated for the mammalian cell monolayer and expressed as a ratio vs. the IC_{50} for that same drug for *Pneumocystis*. Drugs that show little or no toxicity to mammalian cells in the ATP assay are considered for further testing in vivo. We have set the ratio as 1:7 or greater for a compound to be considered for further evaluation. Thus, it must take at least seven times the concentration of a given compound to reduce the ATP by 50% in mammalian cells culture than the concentration of that drug to reduce the ATP of *Pneumocystis* by 50%—e.g., 7 μg in the mammalian cell system vs. 1 μg in the *Pneumocystis* system.

Procedure. Confluent monolayers of the lung cell carcinoma A549 (ATCC CCL, 185), the diploid fibroblast line WI-38 (ATCC CL-75) and the cell line Hep-G2 (ATCC-HB-8065) are established in 24-well plates containing 1 mL of DMEM with high glucose (4.5 g/L) (Fisher Scientific Inc., Cincinnati, OH) 10% fetal bovine serum (Fisher Scientific), 0.1 mM nonessential amino acids, L-glutamine (0.2mM), 1X MEM vitamins, and 1.1 $\mu\text{g}/\text{mL}$ sodium pyruvate (Fisher Scientific). Media containing varying concentrations of each test compound are added to plate wells in triplicate. Triplicate wells are harvested for each time point tested. Medium alone serves as a negative control and antimycin A is used

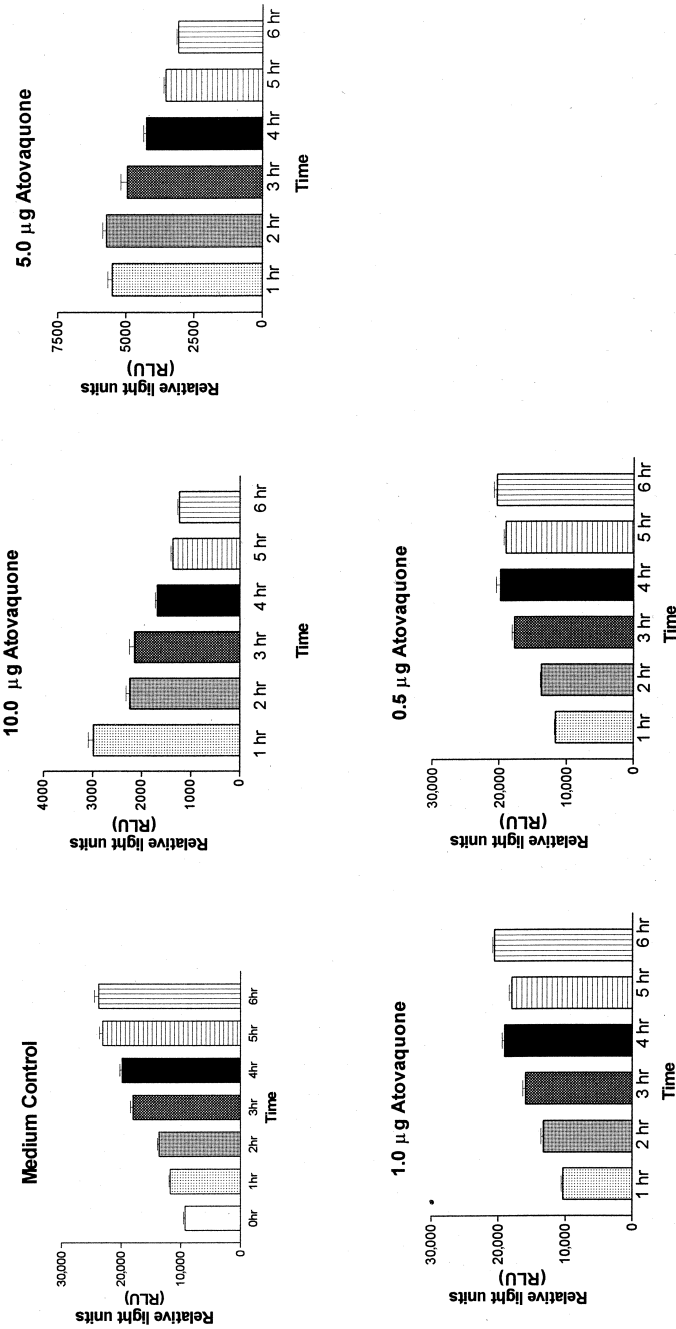


Figure 3 Early responses of *Pneumocystis* to different atovaquone concentrations. ATP levels of *P. carinii* populations exposed to different concentrations of atovaquone over a 6-hr period. "Medium control" were the ATP levels from those organisms receiving no drug; the concentrations ranged from 10.0 to 0.5 µg/mL atovaquone. Data are expressed as relative light units (RLU) on the y axis and are the averages of nine separate readings $\pm$ standard errors of the mean. (From Ref. 152.)

as a positive control. The plates are incubated at 35°C, 5% CO₂. At 24-, 48-, and 72 hr, the medium is aspirated and 1 mL of 3.5% trichloroacetic acid in 2 mM EDTA (pH 1.5) is added to each well. After incubation at room temperature for 10 min, plates are stored at -20°C until completion of the experiment and processed as a batch by thawing to room temperature with agitation. After this step, 5-μL samples from each well are removed and placed directly into individual wells of a 96-well opaque white plate containing 100 μl of buffer (200 mM TRIS, 2.5 mM EDTA, pH 7.75). The plates are read in a FluoSTAR Optima Plate Reader (BMG Labtechnologies, Inc.) as described above.

In one representative assay, three compounds received from the National Cancer Institute (639366 V/3, 658276 Z/1, and 675670 I/2) were found to have marked activity against *Pneumocystis* in vitro, with IC₅₀ values of 305 ng/mL, 714 ng/mL, and 118 ng/mL, respectively. These compounds were then evaluated for their toxicity to A549 cells (Table 7). The A549 IC₅₀ values for these compounds were 6.3 μg/mL for 639366 V/3 (for a 1:21 *Pneumocystis*: A549 efficacy ratio), 13.9 μg/mL for 658276 Z/1 (for a 1: 19 ratio), and 826 ng/mL for 675670 I/2 (1: 7 ratio). Thus, for example, 21 times more of compound 639366 (which reduced the ATP of *Pneumocystis* by 50%) was required to reduce the ATP pools of the A549 cells. All of these compounds would be candidates for further evaluation in vivo.

Drug Combination Studies in Vitro

We have initiated combination drug studies using the ATP system with the drugs atovaquone, proguanil, and cycloguanil. We were interested in these drugs for several reasons. Atovaquone and proguanil have been marketed as a fixed combination (Malarone) that has enhanced antimalarial activity. This combination acts

Table 7 Effects of Drugs on A549 Cells

Time point	24 hr	48 hr	72 hr
Media	160,453	135,173	134,640
Antimycin A 50 μg/mL	179,117 (0)	107,587 (20.5)	46,259 (65.7)
639366 V3 30.5 μg/mL	54,203 (66.3)	37,615 (72.2)	23,691 (82.5)
639366 V3 3.05 μg/mL	108,968 (32.1)	104,634 (22.6)	106,910 (20.6)
639366 V3 305 ng/mL	149,768 (6.7)	135,695 (0)	117,083 (13.1)
658276 Z1 71.4 μg/mL	301 (99.9)	2,646 (98.1)	5,920 (95.6)
658276 Z1 7.14 μg/mL	130,110 (19.0)	117,844 (12.9)	119,098 (11.6)
658276 Z1 714 ng/mL	135,830 (15.4)	125,712 (7.0)	133,725 (0.7)
675670 I2 11.8 μg/mL	246 (99.9)	140 (100)	575 (99.6)
675670 I2 1.18 μg/mL	41,884 (73.9)	70,667 (47.8)	36,070 (73.3)
675670 I2 118 ng/mL	119,676 (25.5)	104,686 (22.6)	147,705 (0)

on two different pathways in the pyrimidine synthesis needed for nucleic acid replication: atovaquone inhibits mitochondrial electron transport, and proguanil is metabolized to cycloguanil, the active form of which inhibits DHFR. Several classes of compounds (e.g., hydroxynaphthoquinones, DHFR inhibitors, sulfonamides, 8-aminoquinolones) active against *Plasmodium* are also active against *Pneumocystis*. Atovaquone, proguanil, and cycloguanil for our studies were kindly provided by Glaxo Wellcome. Dose-effect curves were established for each compound against *Pneumocystis* in the ATP system as described above. Curves were generated for 24-, 48-, and 72-hr time points using the CalcuSyn Software (Biosoft, Cambridge, UK). This software program will produce two graphs for a single drug—the median-effect plot and the dose-effect plot—as well as dose-effect and median-effect tables. The median-effect plot provides an r value that introduces the first line of statistics. The dose-effect plots for the 48-hr time point are shown below in Figure 4. The doses are in $\mu\text{g/mL}$ (x axis) and the inhibitory effect is in decimals (e.g., 1.0 = 100%) (y axis).

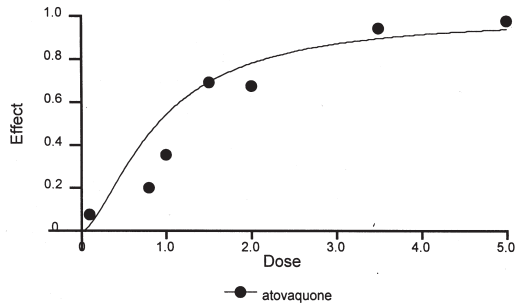
To assess the interactions of the compounds, variable percentages of the IC_{50} s for each compound were added in combination with variable percentages of the IC_{50} s of the other compound. These combinations were set at: 90:10, 75:25, 50:50, 25:75, and 10:90%. The method of assessment of synergy remains controversial and dependent on several considerations: the accuracy of measurement and variability; the dose levels and time points when synergy is to be assessed; the experimental conditions (e.g., pH, oxygen tension may affect data and conclusions); and whether synergy is treatment schedule-dependent or combination ratio-dependent. We have improved the reproducibility of the ATP system by using standardized and characterized “batches” of *Pneumocystis* as inocula and implementation of a measurement system that is based on quantifiable units (photons) rather than organism numbers. This has reduced variability within the system considerably, but we recognize that we are still using a biological system in which stochastic effects are possible. Thus we repeat each experiment (drug series, not wells) at least twice and often in triplicate.

To determine synergy, we have adopted the methods of Chou and Chou and also Chou and Talalay, which have been incorporated into the CalcuSyn Software [170,171]. If the combination index (CI) of two compounds is less than 1, this indicates synergism; if it is equal to 1, the effect is additive; and if the CI is greater than 1, antagonism has occurred. The degree of synergism and antagonism can be further defined by a scale that ranges from <0.1 (very strong synergism) to >10 (very strong antagonism). The CI was calculated for combinations of atovaquone, proguanil, and cycloguanil according to the equation developed by Chou and Talalay:

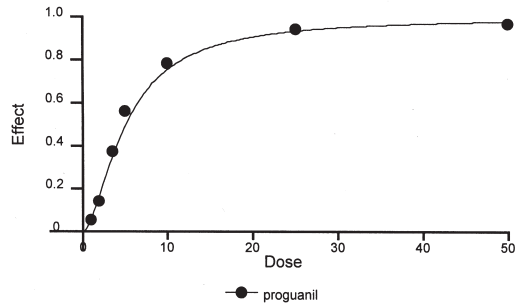
$$\text{CI} = \sum (D)_j$$

$$j = 1 (EDx)_j$$

Atovaquone 48-hr dose effect



Proguanil 48-hr dose effect



Cycloguanil 48-hr dose effect

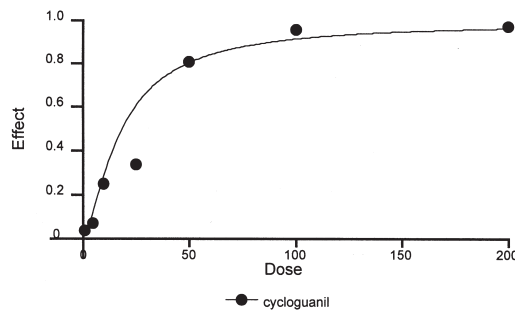


Figure 4 Forty-eight-hour dose response curves for atovaquone, proguanil, and cycloguanil. *P. carinii* populations were treated with varying doses of each drug and evaluated for decreases in ATP levels vs. untreated organism pools. The percent decrease in ATP is expressed on the y axes on a scale from 0 (no effect) to 1.0 (ATP levels at or below background). The doses of the drugs are on the x axes.

Where n is the number of inhibitors; D is the drug effect; and EDx is the effective dose (in our case inhibitory concentration, IC).

Five different graphic representations are available to illustrate the effects of combinations as well as output reports. Monte Carlo estimation can be used to generate the statistical uncertainty measurements in CI tables and plots or estimated using the algebraic approximation. We have chosen to use the Monte Carlo estimation at 1000 iterations, a standard procedure. Graphic output can be (1) median plot effect; (2) dose effect plot; (3) mixture-algebraic estimate; (4) mixture—nonexclusive (for two-drug combinations only); and (5) isobologram (normalized and conservative). For illustration purposes, we show the normalized isobolograms for the drug combinations described in Figure 5. The isobolograms are generated from the CI equation described above. Since our data were in a nonconstant ratio design, conservative isobolograms could not be constructed and the normalized (D_x) were plotted.

These two isobolograms illustrate antagonism (atovaquone/proguanil) and synergism (atovaquone/cycloguanil). All combinations of the atovaquone and proguanil were well above the additive line (1.0 connecting 1.0), suggesting strong antagonism between the compounds. In contrast, all but one of the combinations (1:1) of the atovaquone/cycloguanil were well below the line, suggesting strong synergism. These interactions were the same for the 24- and 72-hr time points. (The ratios listed below each isobologram (e.g. 1:27, 1:9, 1:3) are based on the concentration of each drug added in the combination and not on the ratios of the IC_{50} s.)

The results presented here are preliminary and require further studies for confirmation and interpretation of their significance. Nevertheless, these data suggest that the ATP system can be used to predict synergism, additive effects, and antagonism and thus represent a major advance in our ability to identify candidate drug combinations, not simply single-agent therapies. The selection of drug combinations for in vivo testing is described in the following section.

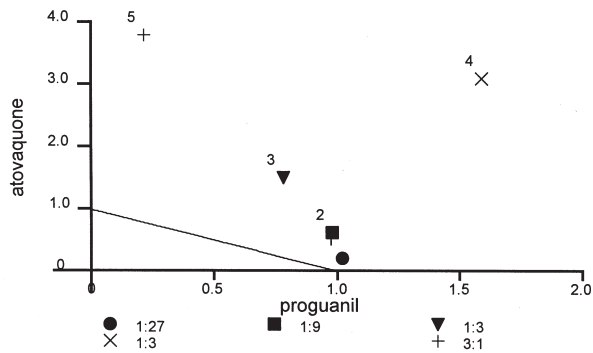
B. Studies in Animal Models

Mice

Source of Animals

We use C3H/HeN mice because these animals are highly susceptible to *Pneumocystis* pneumonia induced by different forms of immunosuppression and exposure to infected animals; these mice also tolerate the immunosuppressive regimens quite well [48]. The mice are obtained from Charles River (Hollister, CA) and have been shown to be free from latent *Pneumocystis* infection by DNA amplification, serological testing, and prolonged immunosuppression in the absence of exposure to sources of *Pneumocystis* [172].

Atovaquone/proguanil normalized isobologram
ANTAGONISM



Atovaquone/cycloguanil normalized isobologram
SYNERGISM

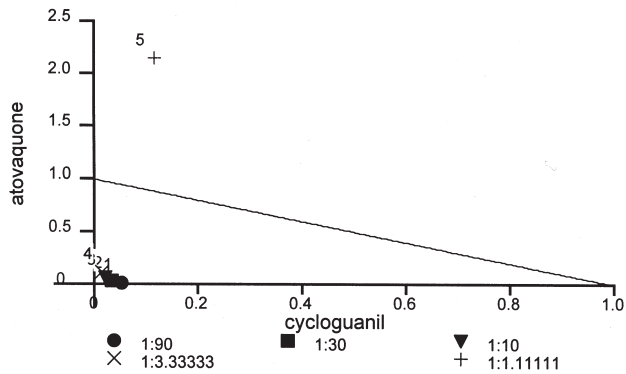


Figure 5 Normalized isobolograms for atovaquone, proguanil, and cycloguanil. Varying ratios of previously determined IC₅₀ concentrations of two drugs were added to *P. carinii* organisms and the decrease in ATP levels of the combinations were determined. The decreases in the ATP levels were evaluated for synergism, antagonism or additive effects based on the Combination Index using the software program CalcuSyn. (From Refs. 170 and 171.)

Source of *Pneumocystis*

Mouse *Pneumocystis* (originally provided by Dr. Allen Harmsen, Trudeau Institute, Saranac Lake, NY) has been serially propagated in our mouse colony by housing mice with active *Pneumocystis* pneumonia in the same cage with naïve immunosuppressed mice (“seeding”)[173]. In order to ensure that equal exposure occurs, the mice are rotated among cages. In selected instances, immunosuppressed mice are inoculated intratracheally with infected lung homogenate. Analysis of *Pneumocystis* from our mice for chromosomal migration by CHEF and of the mitochondrial ribosomal RNA (mtRNA) gene by amplification and DNA sequencing suggested that the mouse *Pneumocystis* is a single isolate. Shown in Figure 6 are examples of *Pneumocystis* CHEF profiles from two individual mouse lungs (mPc1, mPc2), which are identical to one another but distinct from the rat *P. carinii* form 1 profile (rPc). We have not observed the karyotypic diversity observed among populations of rat *Pneumocystis* (13 different profiles) among mouse *Pneumocystis* isolates.

Experimental Protocols

The methods of inducing *Pneumocystis* pneumonia, determining the extent of infection, and evaluating the effectiveness of candidate anti-*Pneumocystis* drugs are very similar in mice and rats. Treatment studies are the most common type

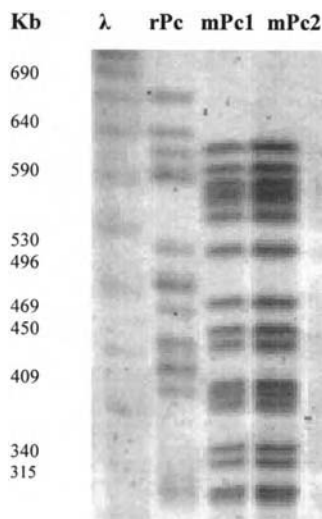


Figure 6 CHEF Analysis of *P. carinii* and mouse-derived *Pneumocystis*. Lane 1: concatamerized lambda genomes in 48.5 kb increments used as size markers, Lane 2: *Pneumocystis* from rat (*P. carinii* karyotype form 1). Lane 3: Mouse *Pneumocystis* from mouse 1. Lane 4: Mouse *Pneumocystis* from mouse 2.

of protocol. C3H/HeN mice weighing about 25 g (Charles River) are placed in microisolator cages and housed at the University of Cincinnati (UC) under barrier conditions, with laminar air flow and HEPA filter, where they are given autoclaved food, water, and bedding. After acclimation for about a week, the mice are administered dexamethasone (Butler, Columbus, OH) 2 mg/500 mL in the drinking water and housed with infected animals to induce *Pneumocystis* infection. An antibiotic such as ampicillin or cephadrine 0.5 mg/mL (Velosef, Bristol-Myers Squibb, Princeton, NJ) is added to the drinking water to prevent the development of bacterial infection. The mice are weighed and monitored, and sentinel animals are sacrificed periodically to check the progression of *Pneumocystis* pneumonia. After about 6 to 7 weeks, when the infection has reached moderate severity, the mice are randomly divided into treatment and control groups of about 10 animals each.

Drugs to be tested are usually dissolved in 2% ethanol and given parenterally or by oral gavage on a milligram-per-kilogram basis in single or divided doses for 3 weeks, during which time they continue on the immunosuppressive regimen. The following control groups are used: control steroid (C/S) mice that are administered no drugs or a placebo; mice administered the standard anti-*Pneumocystis* drug, TMP/SMX 50/250 mg/kg/day PO; and mice without *Pneumocystis* pneumonia administered the drug(s) to be tested if toxicity is being evaluated. The animals are observed daily for appearance, activity, and food and water consumption. Mice that appear ill or moribund are sacrificed by the methods approved by the AAALAC Association for Assessment and Accreditation of Laboratory Animal Care, with an overdose of carbon dioxide or halothane anesthesia; their lungs are processed as described below. If drug toxicity or infection with another opportunistic pathogen is suspected, tissue or culture specimens are obtained from the lungs or other organs as deemed appropriate.

Prophylaxis studies are performed on a more selective basis, usually on agents that have shown activity in the therapy of *Pneumocystis* pneumonia. We are mainly interested in whether low doses of the drug administered for a long period of time can prevent the development of *Pneumocystis* pneumonia and how well these doses are tolerated by the host. The protocol is similar to that for treatment experiments except that the candidate drugs and TMP/SMX, the standard agent, are administered throughout the entire period of immunosuppression. Prophylaxis studies usually last 9 to 10 weeks, and the animals are sacrificed as described above.

Assessment of Drug Efficacy

The evaluation of drug activity is based on reduction in the severity or extent of *Pneumocystis* pneumonia in the lungs rather than animal survival because, as mentioned previously, the animals may die from causes unrelated to *Pneumocystis* (e.g., drug toxicity, opportunistic infections) [5,48,68,86,174]. In the treatment studies,

the animals have to receive the drugs for a minimum specified period of time to be included in the data analysis because we have observed that it usually takes this long to see an effect of drugs on the organism. (Most drugs we have tested for anti-*Pneumocystis* activity act slowly on the organism.) In the past, this period of time was 10 days. However, as our experience using mice to test drugs has grown, we reevaluated this period of time and found that we can detect a drug effect on *Pneumocystis* in mice and rats by 7 days of treatment. While early deaths are not a major problem, changing to 7 days has enabled us to make better use of the animals in an experiment when these deaths occur. The principal way of determining the extent of *Pneumocystis* pneumonia is by quantitating the organism burden in lung homogenates and expressing the result as the mean $\log_{10}$ number $\pm$ standard deviation (SD) organisms per lung [174]. We have found this approach to be simple, rapid, inexpensive, and more sensitive than histology in detecting changes in therapy. The advantages of this approach are that it is analogous to the standard microbiological practice of using quantitative bacterial or fungal cultures; it is a language that any investigator who works with animal models of infectious diseases can easily understand; and it permits direct comparison of the results obtained by one *Pneumocystis* group of investigators with those of another. An alternative approach, which assigns a score of 1+, 2+, 3+, etc., to the estimated number of organisms seen on imprint smears [175], has significant limitations but also the advantage of rapid assessments. Imprint smears from one lung lobe are less sensitive and less accurate than a homogenate from an entire lung in evaluating the organism burden. The numbering system used with imprint smears can detect more dramatic changes in the *Pneumocystis* burden in response to anti-*Pneumocystis* drugs, but more subtle yet significant differences cannot be observed. Moreover, the results from semiquantitative imprint scales are difficult to compare among different groups of investigators due to their somewhat subjective nature.

For *Pneumocystis* quantitation, the right lung is homogenized in a Stomacher Lab Blender (Tekmar, Cincinnati, OH) erythrocytes are lysed with aqueous ammonium chloride (0.85%), washed, centrifuged, and resuspended in 1 mL of phosphate buffered saline. Three 0.01 μ l drops each covering an area of 1 cm² are placed on a glass slide and air-dried. The slides are stained with cresyl echt violet (CEV), which selectively stains *Pneumocystis* cysts, and Diff-QuikTM, a rapid variant of the Wright-Giemsa stain that stains the nuclei of all developmental stages. The slides are coded and read in a blinded manner after which the number of cysts or nuclei per oil immersion field (OIF) is determined by randomly counting 30 OIF (10 fields per drop). This number is multiplied by a conversion factor for the microscope (calculation of area analyzed) and by the dilution to arrive at the total number per lung. The lower limit of detection for our microscope in the analysis of mouse *Pneumocystis* is $\log_{10}$ 4.35 (2.23×10^4) organisms per lung. Cyst quantitation has been performed routinely because it is faster, requires less expertise, and is less tedious than quantitation of nuclei. Our previous experience

in performing chemotherapy studies had been that counts of nuclei are higher than counts of cyst but they added little useful information to what had already been obtained from cyst quantitation. Quantitation of nuclei is performed on a more selective basis (e.g., to confirm the cyst data obtained with a new drug being tested or to determine the effects of a drug on different *Pneumocystis* developmental stages). However, in recent adoptive transfer experiments performed in rats, we have noticed different effects of immune lymphocytes on counts of *Pneumocystis* cysts and nuclei [28]. These results have caused us to reevaluate our view of counts of nuclei to include both microscopic analyses in our evaluation of potential anti-*Pneumocystis* drug candidates.

Drug effectiveness is determined by comparing the organism burden in the treatment groups with the control steroid (C/S) group and the mice treated with TMP-SMX in the same experiment. Statistical analysis on normally distributed data is performed by an analysis of variance (ANOVA) followed by Student's t-test with the Neuman-Keuls correction factor for multiple comparisons using INSTAT (GraphPad Software for Science, San Diego, CA). Nonparametric statistics are used for non-normally-distributed data: the Kruskal-Wallis analysis of variance is used first and followed by the Wilcoxin rank sum test with the Dunn's correction factor for multiple comparisons. In calculating sample size, we have made certain assumptions based on our classification system of the drug's anti-*Pneumocystis* activity in animals (see below)[68,86]. The minimum reduction in mean or median *Pneumocystis* count in this system for a drug to have any activity is fivefold ($\log_{10}0.70$) (see below). If each population has a standard deviation (SD) of $\log_{10} 0.60$, then a minimum sample size of nine mice per group would be required to detect statistically significant differences with the alpha (two-sided) set at 0.05 and a power of 80%. Assuming that some mice die from adverse drug effects or other opportunistic infections before the end of the experiment, we have used at least 10 mice per group as a reasonable sample size.

Our system of classifying the activity of candidate anti-*Pneumocystis* drugs is based on the magnitude of reduction in *Pneumocystis* counts. The categories in the activity scale are as follows: very marked, ≥ 1000 -fold reduction; marked, 100- to 999-fold reduction; moderate, 10- to 99-fold reduction; slight, 5 to 9-fold reduction; and inactive (none), <5 -fold reduction; A major advantage of the classification system is that it allows comparison of drugs or classes of drugs studied at different times and in different experiments.

A second method of analyzing drug efficacy is histopathological examination of the lung. This is performed to confirm the results obtained by organism quantitation, to investigate the host inflammatory response, and to look for evidence of drug toxicity or other opportunistic infections. Sections of the left lung are prepared for histological analyses by fixation in paraformaldehyde and embedding in paraffin [174]. They are then stained with hematoxylin and eosin and methenamine silver, coded, and read in a blinded manner by an experienced

pathologist. A semiquantitative scoring system has been established which is based on the proportion of alveoli involved: 0 (negative); 0.5 +, minimal (>1% alveoli); 1 +, light (1 to 25% alveoli); 2 +, moderate (25 to 50% alveoli); 3 +, severe (50 to 75% alveoli); 4 +, very severe (>75% alveoli). A highly significant correlation has been found between the histological score and the number of *Pneumocystis* cysts or nuclei in lung homogenates [30,174,176].

A third method of evaluating drugs is determining the ratio of lung weight to body weight. This analysis, which is performed on a selective basis, is a less direct measure of drug activity than *Pneumocystis* quantitation or lung histology. This ratio increases with more extensive *Pneumocystis* pneumonia, thus reflecting the organism burden, edema, and cellular infiltration [174]. The ratio of lung weight to body weight is also a measure of the host inflammatory response. In our adoptive transfer studies, CD4 cells sensitized to the major surface glycoprotein of *Pneumocystis* antigen caused a hyperinflammatory response in the recipient animals, which was reflected by an increased ratio of lung weight to body weight and associated with high mortality; those animals that survived cleared *Pneumocystis* from their lungs [128,177].

Rats

Source of Animals and *Pneumocystis*

Lewis rats weighing about 125 to 150 g (Charles River) are housed at the Cincinnati VAMC under barrier conditions similar to those described for mice above. The predominant isolate in our rat colony is *P. carinii* form 1; the same used in our in vitro studies. *Pneumocystis* is propagated in the colony mainly by housing rats that have *Pneumocystis* pneumonia with other immunosuppressed rats. Occasionally intratracheal inoculation is used.

Experimental Protocols

After acclimization, the rats are administered methylprednisolone acetate (Depo-Medrol, UpJohn Pharmacia, Kalamazoo, MI) 4 mg/kg subcutaneously once weekly and housed with *Pneumocystis*-infected animals to produce *Pneumocystis* pneumonia. Other aspects of the experimental protocols as well as the methods used to analyze and classify drug activity are the same as those used in mice.

Drugs Analyzed

Selection of Compounds for Testing. As described in our flow diagram (Fig. 1), compounds that show marked or very marked anti-*Pneumocystis* activity and little or no toxicity in vitro are candidates for testing in vivo. The major limiting factor here is whether sufficient quantities of these agents are available for studies in our mouse model. Unfortunately, more often than not, these quantities are not available. The major reason for this problem is that these drugs

represent newly synthesized compounds and are either difficult to make or the investigator who made them does not have the resources to produce larger amounts. NIH has a mechanism to synthesize large amounts of drugs but has to be quite selective in its choice of agents. Factors that influence this process include the compound's physical and chemical properties, problems for large-scale production, activity against other opportunistic pathogens, toxicity, and route of administration.

An alternative strategy we have used to select compounds for in vivo testing is to examine drugs that are already used clinically for other purposes to see if they have activity against *Pneumocystis*. One advantage of this approach is that there are unlimited quantities of the agent available for use in our animal models. We usually combine our in vivo studies with in vitro evaluation of the properties of these agents. The agents discussed here illustrate the following points: the method of selection for testing in vivo; the correlation between in vitro and in vivo results; the correlation between mouse and rat models of testing; and the application of our results to clinical medicine.

Anti-HIV Drugs. Our studies in the early 1990s had shown that nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine or didanosine displayed little or no anti-*Pneumocystis* activity in our rat model of *Pneumocystis* pneumonia [65]. However, the introduction of highly active antiretroviral therapy (HAART) with agents such as protease inhibitors led to a dramatic fall in the incidence of *Pneumocystis* pneumonia and other opportunistic infections. While the success of the protease inhibitors has been thought to be due to their effects on HIV and the host's immune system, recent studies suggest these agents act directly against *Pneumocystis* and other fungi such as *Candida albicans* [178–180]. Like HIV, these organisms contain aspartyl proteases that may be important virulence factors; the protease inhibitors inhibited growth in vitro at concentrations achievable in human serum. These reports, which stimulated clinical and media interest [181] have important implications: they extend the potential use of the protease inhibitors well beyond their value as antiviral drugs; they raised the possibility of a new class of anti-*Pneumocystis* drugs already licensed for clinical use. Since *Pneumocystis* drug development has received little interest from the pharmaceutical industry, the testing of clinically available drugs for activity against the organism represents an alternative approach to finding new agents.

The report of anti-*Pneumocystis* activity of the protease inhibitors was based on in vitro susceptibility testing using cell culture [180]. Before clinical application of these results is considered, we felt that the studies should be repeated by other investigators using a different in vitro technique and in an established animal model [182]. In our first set of studies, we examined 12 anti-HIV drugs using the ATP assay. The results showed that of the protease inhibitors indinavir (IC₅₀

35.4 $\mu\text{g/mL}$) and saquinavir (IC_{50} 23.3 $\mu\text{g/mL}$) exhibited slight anti-*Pneumocystis* activity; however, these drug levels exceeded those that can be clinically achieved in serum [183–185]. Amprenavir or ritonavir showed no activity, with IC_{50} values far above 100 $\mu\text{g/mL}$, the highest level tested. Similarly, no anti-*Pneumocystis* activity was found in the NRTIs (abacavir, didanosine, lamivudine, stavudine, zalcitabine, or zidovudine) or nonnucleoside reverse transcriptase inhibitors (NNRTIs) (delavirdine, nevirapine) that were tested. Pentamidine (2 $\mu\text{g/mL}$), the positive control drug, exhibited excellent activity (IC_{50} 0.29 $\mu\text{g/mL}$) against the organism, whereas ampicillin (10 $\mu\text{g/mL}$), the negative control drug, did not ($\text{IC}_{50} > 100 \mu\text{g/mL}$).

Our first in vivo experiment focused on protease inhibitors (Table 8). None of the drugs reduced the mean *Pneumocystis* cyst count significantly below that in the control mice. Indinavir at a dose of 200 mg/kg/day lowered the mean organism count about sixfold, a level that was categorized as slight on our classification scale; saquinavir and nelfinavir reduced the cyst count by about threefold. Another experiment analyzed combinations of NTRIs, NNTRIs, and protease inhibitors (Table 9). None of the regimens exhibited anti-*Pneumocystis* activity. By contrast, TMP-SMX, the standard positive control drug, lowered the cyst count by 2512-fold and 69-fold, respectively, in the two studies. All drugs were well tolerated by the mice.

Taken together, our data suggest that anti-HIV drugs have little or no activity against *Pneumocystis* in vitro or in vivo. The reasons for the disparate results of our study and those in the report cited above [180] are unclear. One possible explanation is that there are species and/or strain differences in the rat *Pneumo-*

Table 8 Treatment of *Pneumocystis* Pneumonia in Mice with HIV Protease Inhibitors

Group	Treatment	Dose regimen	No. of Mice	<i>Pneumocystis</i> Count	<i>p</i> Value
1	C/S ^a	—	12	7.87 $\pm$ 0.40	—
2	Indinavir	50 mg/kg/day PO	12	7.90 $\pm$ 0.34	N/S
3	Indinavir	200 mg/kg/day PO	11	7.10 $\pm$ 1.05	N/S
4	Nelfinavir	50 mg/kg/day PO	12	7.38 $\pm$ 0.49	N/S
5	Nelfinavir	200 mg/kg/day PO	9	7.91 $\pm$ 1.00	N/S
6	Saquinavir	200 mg/kg/day PO	12	7.36 $\pm$ 0.78	N/S
7	Amprenavir	50 mg/kg/day PO	12	8.32 $\pm$ 0.48	N/S
8	Amprenavir	200 mg/kg/day PO	10	8.38 $\pm$ 0.28	N/S
9	Lopinavir/ritonavir	50/16.6 mg/kg/day PO	11	7.59 $\pm$ 0.79	N/S
10	Lopinavir/ritonavir	200/66.6 mg/kg/day PO	10	7.86 $\pm$ 0.97	N/S
11	TMP-SMX ^b	50/250 mg/kg/day PO	7	4.47 $\pm$ 0.32	<0.001

^a Control steroid.

^b Trimethoprim-sulfamethoxazole.

Table 9 Treatment of *Pneumocystis* Pneumonia in Mice with Anti-HIV Drugs

Group	Treatment	Dose regimen	No. of mice	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	10	7.29 ± 0.92	N/S
2	Nevirapine/stavudine	5/10 mg/kg/day PO	12	7.24 ± 1.12	N/S
3	Nevirapine/stavudine/ritonavir	5/10/180 mg/kg/day PO	10	7.32 ± 0.41	N/S
4	Delavirdine/stavudine	150/10 mg/kg/day PO	10	7.02 ± 1.50	N/S
5	Delavirdine/stavudine/ritonavir	150/10/180 mg/kg/day PO	10	7.31 ± 0.52	N/S
6	Stavudine/lamivudine/ritonavir	10/150/180 mg/kg/day PO	8	6.96 ± 1.30	N/S
7	Ritonavir	180 mg/kg/day PO	10	6.99 ± 1.06	N/S
8	TMP-SMX ^b	50/250 mg/kg/day PO	7	5.45 ± 1.60	<0.05

^a Control steroid.^b Trimethoprim-sulfomethoxazole.

cystis isolates used as test organisms and that these are reflected in the susceptibility to the protease inhibitors. We routinely use *P. carinii* form 1 for in vitro drug work; since the isolate was not characterized in the other report, a direct comparison is not possible. However, as mentioned previously, we have found no differences in drug susceptibility in vitro among genetically different strains of rat *Pneumocystis* we have tested. Another possibility involves differences in the in vitro systems: the ATP assay is a cell-free system that measures *Pneumocystis* by ATP content, whereas the Spinner flask cell culture system counts *Pneumocystis* developmental stages stained with Giemsa stain. It is also possible that a drug used in cell culture could exert its effect on *Pneumocystis* indirectly through its effect on the cell monolayer. A third possibility involves biochemical or metabolic differences within the milieu of the culture systems. HIV protease inhibitors inhibit the growth of *C. albicans* under nitrogen-limited conditions but not on standard fungal media (e.g., Sabouraud, YPD) [178].

Our results are important because they emphasize the need for caution in interpreting *Pneumocystis* drug susceptibility data based only on in vitro testing, particularly when cell culture is the technique used. The doses of the HIV drugs we used in mice were similar those used in other animal models [186,187]. The strong correlation between our in vitro and in vivo data strengthens confidence in our findings and conclusions.

Streptogramins. Quinupristin dalfopristin (Q/D) (Synercid) is a fixed combination of semisynthetic streptogramins that has been marketed primarily for the treatment of serious resistant enterococcal infections. Q/D is active against most gram-positive aerobic and anaerobic bacteria; some gram-negative bacteria (e.g., *Moraxella catarrhalis*, *Neisseria* spp., *Legionella* spp., *Mycoplasma* spp., *Chlamydia* spp.) that cause respiratory infections; and the protozoan parasite *Toxoplasma gondii* [188,189]. Since some drugs that have anti-*T. gondii* activity

are also active against *Pneumocystis*, we decided to examine the effects of Q/D by our in vitro and in vivo systems [113].

Q/D reduced the ATP pools in *Pneumocystis* in a dose- and time-dependent manner. The maximal effect was seen at 72 hr, with an IC_{50} of 10.6 $\mu\text{g/mL}$, which amounts to slight to moderate activity on our scoring scale. The toxicity of Q/D was determined by analyzing its effects on the ATP pools of A549 cells. The IC_{50} of the drug for A549 cells was 122.0 $\mu\text{g/mL}$ after 72 hr of incubation, compared with an IC_{50} of 10.6 $\mu\text{g/mL}$ for *Pneumocystis* at the same time point, a greater than tenfold difference (Fig. 7).

QD administered at doses of 50 and 100 mg/kg/day in the treatment of *Pneumocystis* pneumonia in our mouse model lowered the mean organism count four- to five fold (slight activity) from $\log_{10} 7.49 \pm 0.57$ per lung in the C/S group to 6.82 ± 0.63 per lung and 6.87 ± 0.37 per lung, respectively ($p < 0.01$) (Table 10, Experiment 1). QD at a dose 200 mg/kg/day reduced the organism count 15-fold (moderate activity) to 6.32 ± 0.46 per lung ($p < 0.001$). This was greater than the reduction with the 50 or 100 mg/kg/day doses ($p < 0.05$). TMP-SMX given at a dose of TMP 50mg to SMX 250mg/kg/day lowered the organism count 851-fold (marked activity), to 4.56 ± 0.38 per lung ($p < 0.001$). This was greater than that achieved with the dose of Q/D of 200 mg/kg/day ($p < 0.001$).

Nonparametric statistics were used to analyze the data for the prophylaxis experiment because the data were not normally distributed (Table 10, Experiment 2). Q/D administered at a dose of 100 mg/kg/day IP 3 times per week decreased the median *Pneumocystis* count 302-fold (marked activity), from 8.01 per lung in the C/S group to 5.53 per lung ($p < 0.01$). For comparison purposes, TMP-SMX given at a dose of TMP 50 mg to SMX 250 mg/kg/day as treatment during

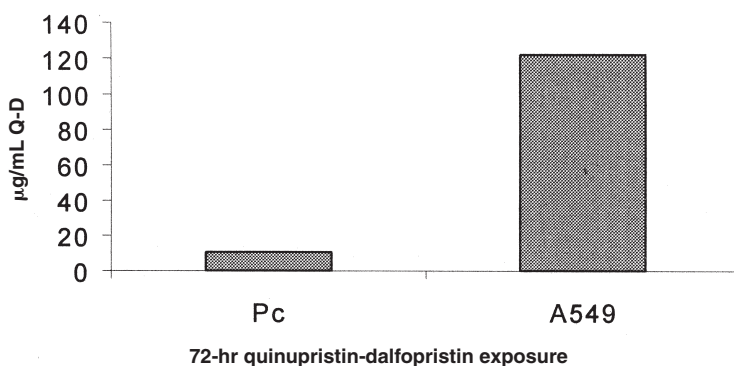


Figure 7 IC_{50} for *Pneumocystis* vs. A549 cell monolayers after 72 hr of quinupristin-dalfopristin exposure. The IC_{50} of the drug for *P. carinii* was 10.6 $\mu\text{g/mL}$ compared with an IC_{50} of 122.0 $\mu\text{g/mL}$ for A549 cells. (From Ref. 114.)

Table 10 Treatment and Prophylaxis of *Pneumocystis* Pneumonia in Mice with Quinupristin-Dalfopristin

Group	Treatment	Dose regimen	No. of mice	<i>Pneumocystis</i> count	<i>p</i> Value
Experiment 1					
1	C/S	—	16	7.49±0.57	—
2	Q/D	50 mg/kg/day IP	8	6.82±0.63	<0.01
3	Q/D	100 mg/kg/day IP	10	6.87±0.37	<0.01
4	Q/D	200 mg/kg/day IP	11	6.32±0.46	<0.001
5	TMP-SMX	50/250 mg/kg/day PO	11	4.56±0.38	<0.001
Experiment 2					
1	C/S	—	11	7.95±0.47	—
2	Q/D	100 mg/kg/3x/week IP	10	5.53±1.10	<0.001
3	TMP-SMX	50/250 mg/kg/day PO	8	4.35±0.00	<0.001

Key: C/S, control steroid; Q/D, quinupristin/dalfopristin; TMP-SMX, trimethoprim-sulfamethoxazole.

the last 3 weeks of the study drug lowered the median organism count 4570-fold (very marked activity) to undetectable levels (≤ 4.35 per lung) ($p < 0.001$).

Q/D caused transient, dose-related lethargy in the mice for the first few days but was otherwise well tolerated in both experiments. Q/D given at a dose of 500 mg/kg/day IP resulted in the death of the mice within minutes after administration.

The in vitro data presented here suggest that Q/D was less active against *Pneumocystis* than against *T. gondii* in tissue culture [189] and also less active than standard anti-*Pneumocystis* drugs against the organism in the ATP assay [150]. However, the activity of Q/D is similar to that of some inhibitors of sterol biosynthesis, which are potential *Pneumocystis* drug targets [162]. Q/D administered to humans at higher than clinically used doses (12.6 to 29.4 mg/kg) achieves serum levels (10.7 to 24.2 $\mu\text{g/mL}$) that exceed the IC_{50} of 10.6 $\mu\text{g/mL}$ [190]. The slight to moderate activity of Q/D in the treatment of established *Pneumocystis* pneumonia in mice correlated well with our in vitro data. Q/D was much more effective in *Pneumocystis* prophylaxis—a finding that is consistent with our previous experience of studying drugs in this manner [5,68]. Our results with Q/D were obtained in a model of chronic infection, whereas most studies of the drug have been performed in models of acute infection [188]. However, since Q/D given subcutaneously at a dose of 120 mg/kg results in a serum level of 13.2 $\mu\text{g/mL}$ [191], seems likely that we achieved serum levels that exceed the IC_{50} for *Pneumocystis* with our intraperitoneal doses of 100 to 200 mg/kg of the drug.

Judging from its IC_{50} and need for parenteral administration, it appears unlikely that Q/D will be developed for its anti-*Pneumocystis* properties in humans. However, our data are important because they extend studies of Q/D to a

new organism and new type of animal model. More potent anti-*Pneumocystis* agents may be discovered among other streptogramins; with the aid of the *Pneumocystis* genome project [192,193], this may bring new insights about the mechanism of drug action. There may also be increased understanding of other interesting properties of Q/D, such as its short half-life and long postantibiotic effect, concentration in macrophages, and suppression of cytokine responses.

Allylamines. Terbinafine, a member of the allylamines that have been marketed for the treatment of onychomycosis, also has activity against dermatophytes, other fungi, and trypanosomes [194]. The target of the allylamines is squalene epoxidase, the second step in the sterol biosynthetic pathway (Fig. 2). Two studies have shown that terbinafine is active against rat and human *Pneumocystis* at concentrations of 300 $\mu\text{g/mL}$ and 0.4 to 0.8 $\mu\text{g/mL}$, respectively, in tissue culture [112,167]. The investigators also found that terbinafine given at oral doses of 15 to 80 mg/kg/day had efficacy that was equal to or greater than that of known anti-*Pneumocystis* drugs [111,112]. These provocative reports led to interest in possibly using terbinafine to treat *Pneumocystis* pneumonia in humans. As in the case of the anti-HIV drugs discussed above, we decided to see whether we could confirm the claims made for terbinafine. Using the ATP cytotoxicity assay, we found that terbinafine has an IC_{50} of 3.7 $\mu\text{g/mL}$ against rat *Pneumocystis* after 72 hr of exposure [162]. Although this concentration indicates moderate activity on our rating scale, it exceeds serum levels of terbinafine that can be achieved in humans (1 to 2 $\mu\text{g/mL}$) or rodents (2 to 2.5 $\mu\text{g/mL}$) with oral administration of the drug [194,195].

We then evaluated terbinafine in mice with *Pneumocystis* pneumonia [196]. In the first experiment, terbinafine was administered at doses of 20 to 150 mg/kg/day, which were similar to those used by other investigators to treat mouse systemic fungal and protozoal infections (Table 11, Experiment 1). Terbinafine exhibited no anti-*Pneumocystis* activity. By contrast, TMP-SMX lowered the mean organism count by 851-fold (marked activity), which was highly significant. In the second experiment, terbinafine given at a dose of 400 mg/kg/day resulted in a *Pneumocystis* burden that was not significantly different from that in the C/S group (Table 11, Experiment 2). TMP-SMX lowered the count by 2512-fold (very marked activity). The third experiment was performed in **rats** with *Pneumocystis* (Table 12). Terbinafine given at 50 or 250 mg/kg/day produced cyst counts that were not significantly different from those in the C/S group. In contrast, TMP-SMX reduced the count by 1349-fold (very marked activity).

These studies have shown that terbinafine is ineffective as treatment in our mouse and rat models of *Pneumocystis* pneumonia. While terbinafine is active in vitro against fungi that cause systemic infections, it has not been effective as therapy in animal models of these infections [195,197,198]. Following oral administration, terbinafine binds to the stratum corneum, dermis-epidermis, sebum, hair, and nails, where it achieves concentrations higher than those in

Table 11 Treatment of *Pneumocystis* Pneumonia in Mice with Terbinafine

Group	Treatment	Dose regimen	No. of mice	<i>Pneumocystis</i> count	<i>p</i> Value
Experiment 1					
1	C/S ^a	—	16	7.49±0.57	—
2	Terbinafine	20 mg/kg/day PO	11	7.68±0.42	N/S
3	Terbinafine	50 mg/kg/day PO	11	7.65±0.28	N/S
4	Terbinafine	150 mg/kg/day PO	9	7.72±0.51	N/S
5	TMP-SMX ^b	50/250 mg/kg/day PO	11	4.56±0.38	<0.001
Experiment 2					
1	C/S	—	12	7.87±0.40	—
2	Terbinafine	400 mg/kg/day PO	12	7.16±0.98	N/S
3	TMP-SMX	50/250 mg/kg/day PO	7	4.47±0.32	<0.001

^a Control steroid.^b Trimethoprim-sulfamethoxazole.

plasma [194]. Yet even when terbinafine reached a concentration in the lungs of about 6 µg/mL after parenteral administration, the drug was ineffective in the treatment of experimental pulmonary aspergillosis [197].

As with anti-HIV drugs, a possible explanation for the conflicting results with terbinafine reported here and by investigators mentioned above is the presence of species/strain differences in *Pneumocystis* infecting the animal colonies. In addition to their terbinafine data, these investigators reported findings (the effectiveness of oral pentamidine, relative lack of efficacy of oral atovaquone and albendazole) that are at variance with studies by other workers. Another possible reason involves differences in absorption of terbinafine. Early studies that used nonclinical preparations of terbinafine in experimental infections showed that serum levels of the drug were influenced by the solvent used [195]. Both groups here used the clinical formulation of the drug that is well absorbed.

Table 12 Treatment of *Pneumocystis* Pneumonia in Rats with Terbinafine

Group	Treatment	Dose regimen	No. of rats	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	14	8.29±0.30	—
2	Terbinafine	50 mg/kg/day PO	14	7.70±1.15	N/S
3	Terbinafine	250 mg/kg/day PO	9	7.66±0.76	N/S
4	TMP-SMX ^b	50/250 mg/kg/day PO	8	5.16±0.25	<0.001

^a Control steroid.^b Trimethoprim-sulfamethoxazole.

Although there were differences (saline vs. ethanol) in solvents, these seem unlikely to account for the different results. If anything, the larger doses of terbinafine in our study should have resulted in higher serum levels. A final possibility involves the duration of terbinafine administration. The group reporting success started the drug at week 4 of immunosuppression and continued it for at least 5 weeks [111,112]. We began the terbinafine at week 7 and continued it for 3 weeks. Taken together, the studies performed on terbinafine illustrate the complexities of *Pneumocystis* drug testing and emphasize the need for the results obtained by one group to be confirmed by others before considering studies in humans.

Other Studies in Our Animal Models

Relationship of Inoculum Size to *Pneumocystis* Pneumonia and Treatment

We conducted several experiments to determine how many organisms it takes to establish *Pneumocystis* pneumonia in immunosuppressed rats by intratracheal inoculation [199]. Rats inoculated with 10^0 , 10^3 , and 10^6 *Pneumocystis* cysts developed *Pneumocystis* pneumonia about 10 weeks later with organism burdens (*Pneumocystis* cyst counts) of 6.89 ± 1.38 per lung, 7.97 ± 0.46 per lung, and 8.56 ± 0.71 per lung, respectively. *Pneumocystis* counts of nuclei were about tenfold higher (data not shown). These results showed that it takes very few organisms to establish *Pneumocystis* pneumonia. We then conducted studies to determine whether inoculum size has an effect on drug efficacy. The agents tested were TMP-SMX and two 8-aminoquinolones obtained through the NIH contract process for evaluation. The first experiment compared inocula of 10^3 cysts (Table 13) and 10^6 cysts (Table 14) and the effects of most drug doses.

Table 13 Treatment of *Pneumocystis* Pneumonia in Rats Inoculated with 10^3 *Pneumocystis* Cysts

Group	Treatment	Dose regimen	No. of rats	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	10	6.65 ± 1.00	—
2	Aminoquinolone A	0.1 mg/kg/day PO	11	5.43 ± 0.59	<0.001
3	Aminoquinolone A	0.3 mg/kg/day PO	11	5.05 ± 0.00	<0.001
4	Aminoquinolone A	1.0 mg/kg/day PO	11	5.05 ± 0.00	<0.001
5	Aminoquinolone B	0.1 mg/kg/day PO	12	5.13 ± 0.19	<0.001
6	Aminoquinolone B	0.3 mg/kg/day PO	10	5.35 ± 0.95	<0.001

Table 14 Treatment of *Pneumocystis* Pneumonia in Rats Inoculated with 10^6 *Pneumocystis* Cysts

Group	Treatment	Dose regimen	No. of rats	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	15	7.15 ± 0.74	—
2	Aminoquinolone A	0.1 mg/kg/day PO	11	5.46 ± 0.20	<0.001
3	Aminoquinolone A	0.3 mg/kg/day PO	11	5.05 ± 0.00	<0.001
4	Aminoquinolone A	1.0 mg/kg/day PO	12	5.05 ± 0.00	<0.001
5	Aminoquinolone B	0.1 mg/kg/day PO	12	5.80 ± 0.81	<0.001
6	Aminoquinolone B ^a	0.3 mg/kg/day PO	10	5.17 ± 0.08	<0.001

^a Control steroid.

The second study compared inocula of 10^3 cysts (Table 15) and 10^6 cysts (Table 16) in the remaining drug doses.

In the first experiment (Table 13 and 14), the organism burden was again higher with the higher inoculum, but the drugs were very effective when either inoculum was used to establish *Pneumocystis* pneumonia. Thus, the 8-aminoquinolones were active in rats as well as mice. In the second experiment (Table 15 and 16), the organism burdens achieved with the different inocula were almost identical. Again, the 8-aminoquinolones and TMP-SMX were highly effective. Overall, these data suggest that inoculum size does not influence the effectiveness of anti-*Pneumocystis* drugs.

Table 15 Treatment of *Pneumocystis* Pneumonia in Rats Inoculated with 10^3 *Pneumocystis* Cysts

Group	Treatment	Dose regimen	No. of rats	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	5	7.37 ± 1.48	—
2	Aminoquinolone B	1.0 mg/kg/day PO	7	5.65 ± 0.77	<0.05
3	TMP-SMX ^b	50/250 mg/kg/day PO	7	5.49 ± 0.74	<0.05

^a Control steroid.^b Trimethoprim-sulfamethoxazole.

Table 16 Treatment of *Pneumocystis* Pneumonia in Rats Inoculated with 10^6 *Pneumocystis* Cysts

Group	Treatment	Dose regimen	No. of rats	<i>Pneumocystis</i> count	<i>p</i> Value
1	C/S ^a	—	6	7.50 ± 0.83	—
2	Aminoquinolone B	1.0 mg/kg/day PO	8	5.27 ± 0.47	<0.001
3	TMP-SMX	50/250 mg/kg/day PO	5	5.45 ± 0.59	<0.001

^a Control steroid.

^b Trimethoprim-sulfamethoxazole.

V. Conclusion

Despite advances in the treatment of HIV, *Pneumocystis* remains a leading cause of pneumonia and mortality in HIV patients. Currently available anti-*Pneumocystis* drugs are limited by significant problems of efficacy, toxicity, and emerging resistance. Because there is little private-sector interest in developing new agents, the NIH has sponsored contracts for drug development since 1984. We have been a contractor in this program since its inception and have been the sole contractor since 1992. We have developed an organized and efficient approach to *Pneumocystis* drug development that not only meets the needs of these contracts but also has the flexibility for innovation. Drugs evaluated for anti-*Pneumocystis* activity include newly synthesized compounds submitted through the project officer or that arise from collaborative research; there are also agents that have already been licensed for the treatment of conditions other than *Pneumocystis* pneumonia. The process begins with the ATP bioluminescent assay, which uses a well-characterized rat *Pneumocystis* isolate as the test organism. The ATP assay provides rapid in vitro screening, offers clear advantages over cell monolayer systems, is semiautomated, can examine drug interactions, and has been adapted to measure toxicity of the drugs to mammalian cells using the A549 lung carcinoma and other cell lines. Compounds that exhibit high activity against *Pneumocystis* and low toxicity are then evaluated in our standard animal model: mice that reliably develop extensive *Pneumocystis* pneumonia by natural environmental exposure and corticosteroid immunosuppression. The most promising compounds in mice are then tested in the traditional animal model—immunosuppressed rats with *Pneumocystis* pneumonia. A high degree of correlation has been found between results obtained in vitro and in our mouse and rat models. Based on the techniques we have developed, we are able to infect animals by natural exposure or intratracheal inoculation; classify drugs in vitro and in vivo on the basis of their anti-*Pneumocystis* activity; determine the optimal dose and route of administration

of promising agents; monitor drug toxicity, the stability of our *Pneumocystis* isolate(s), and the presence of other opportunistic infections; and study drug combinations and interactions.

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