Diagnostic and Therapeutic Advances in Hematologic Malignancies

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edited by

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Preface

There is no field of medicine in which advances in therapy have been so closely linked to a better understanding of molecular medicine than in the area of hematologic malignancies. For example, recent insights into the understanding of Epstein–Barr virus have led to new treatment options for patients with posttransplant lymphoproliferative disorders, as discussed in the chapter by Dr. Richard Ambinder et al. Similarly, Drs. Slack and Gallagher discuss the explosion of recent information regarding the molecular pathogenesis of acute promyelocytic leukemia. This particular morphologic subtype of acute myeloid leukemia warrants separate discussion because of our increased understanding of the pathogenesis of leukemia, as well as the dramatic advances in outcome that have occurred with differentiation therapy provided by the vitamin A derivative all-*trans* retinoic acid, as discussed in the chapter by Drs. Frankel and Powell. New approaches in the therapy of diffuse aggressive lymphomas in relationship to prognostic factors are discussed by Drs. Koc and Schenkein. Novel approaches to cutaneous T-cell lymphomas are discussed by Drs. Foss and Kuzel. Drs. Fonseca and Greipp discuss prognostic factors in myeloma, which are potentially important since they may serve to identify patients who may benefit from aggressive therapy such as bone marrow transplantation, which is discussed in the chapter by Dr. David Vesole.

State-of-the-art reviews are provided in the chapters on AIDS-related non-Hodgkin's lymphoma by Drs. Volm and Von Roenn, adult acute lymphoblastic leukemia by Drs. O'Connor and Weiss, and myelodysplastic syndromes by Drs. Kouides and Bennett, and a new pathologic classification of non-Hodgkin's lymphomas is discussed by Drs. Finn and Kroft. Patients with AIDS-related lymphomas, acute lymphoblastic leukemia, and myelodysplastic syndromes are often particularly difficult to treat, and therapeutic strategies are constantly evolving.

Biologic approaches may be applicable to a variety of hematologic malignancies. These are thoroughly discussed in the chapters by Drs. Dutcher and Wiernik and by Drs. Sternas, Weeks, and Kwak, where updates on anti-idiotype therapy are found.

Considerable controversy in the therapy of patients with hematologic malignancies remains. The use of hematopoietic growth factors in acute myeloid

leukemia is comprehensively addressed in the chapter by Drs. Rowe and Liesveld. Although some studies have shown an improvement in the time to neutrophil recovery and a decrease in the incidence of infection, the addition of hematopoietic growth factors has not yet been formally translated into an improved survival.

We hope that this compendium will provide both the practicing hematologist–oncologist and the academic clinician with a better understanding and a sense of excitement about evolving concepts in hematologic malignancies.

Martin S. Tallman, M.D.
Leo I. Gordon, M.D.

1. New classifications for non-Hodgkin's lymphoma

William G. Finn and Steven H. Kroft

For decades, attempts to design clinically and biologically relevant classifications of non-Hodgkin's lymphoma have been the topic of intense debate [1–5]. In recent years, our understanding of the basic biological aspects of malignant lymphoma has accelerated rapidly. Unfortunately, this explosion of knowledge has imparted extreme complexity to the subject of lymphoma classification. Much of this complexity reflects the true biologic diversity of lymphoid neoplasia. At times, however, this complexity has been aggravated by variation in interobserver reliability [6] and by the use of different terms assigned by different investigators to describe biologically identical cell types or disease entities [4,6]

The evolution of lymphoma classification schemes has paralleled not only our basic understanding of immune system biology but also our ability to clinically manage previously troublesome lymphomas. Traditionally, many low-grade lymphomas were treated similarly — as indolent but incurable diseases best managed by observation or by noncurative therapy with oral antineoplastic agents. However, the recent discovery of novel therapeutic agents and new applications of existing therapies have greatly increased our ability to treat indolent lymphomas with curative intent. Since the response to emerging therapies varies among the indolent lymphomas, the delineation of specific clinicopathologic entities among these lymphomas has taken on greater importance.

This chapter will outline the principles upon which non-Hodgkin's lymphoma classifications are based, and how current approaches to classification have been affected by recent biological and clinical discoveries. The evolution of modern approaches to lymphoma classification will be illustrated by examples of recently recognized categories of lymphoid neoplasia.

Historical perspective — early classifications of non-Hodgkin's lymphoma

Rappaport classification

By necessity, early approaches to the classification of malignant lymphoma were based almost exclusively on the microscopic anatomy and cytology of

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Table 1. Rappaport classification of non-Hodgkin's lymphoma

Nodular	Diffuse
	Lymphocytic, well differentiated
	Lymphocytic, poorly differentiated
	Mixed cell (lymphocytic–histiocytic)
	Histiocytic
	Undifferentiated

Adapted from Rappaport [8].

specific lymphoid tumors, since the immunophenotypic and molecular genetic studies currently in common use were not available. The most widely used of these morphologic classification schemes was originally proposed by Rappaport in the 1950s [7], with subsequent revision [8]. Based on histologic architecture, lymphomas were divided into nodular and diffuse categories. From there, Rappaport's original classification scheme divided non-Hodgkin's lymphoma into two broad categories: histiocytic lymphoma (reticulum cell sarcoma) and lymphocytic lymphoma (lymphosarcoma). The lymphocytic lymphomas were then subdivided according to their presumed degree of biologic differentiation (undifferentiated, poorly differentiated, well differentiated) (Table 1). The term *histiocytic lymphoma* of Rappaport is no longer used, since these tumors have been shown to be phenotypically lymphoid and not histiocytic. *True histiocytic lymphoma* is a term now used only for those very rare tumors displaying true histiocytic differentiation.

Despite the lack of available immunologic, cytogenetic, and molecular genetic criteria, the Rappaport classification maintained remarkable clinical significance when it first came into usage. For example, nodular lymphomas tended to exhibit indolent behavior in comparison to diffuse lymphomas. Furthermore, the lymphocytic lymphomas (well-differentiated and poorly differentiated) were clinically indolent in comparison to the undifferentiated and histiocytic lymphomas [9].

Immunologic and ontogenic models of lymphoma classification: Kiel, and Lukes–Collins

In the 1970s, rapid advances in the basic understanding of the immune system also led to new approaches for the classification of lymphoid neoplasms. The classification of Lukes and Collins [10] and the Kiel classification [11] employed both morphology and emerging concepts of immune physiology to classify malignant lymphoma based upon the postulated relationship between the neoplastic lymphoid cells and their normal counterparts in the immune system (Table 2). Thus the concept of nodular lymphoma by the Rappaport system was replaced by the concept of follicular lymphoma, implying a lymphoma that mimicked the architectural differentiation of the normal lym-

Table 2. Lukes–Collins classification of non-Hodgkin's lymphoma as originally proposed in 1974

I.	U cell (undefined cell) type
II.	T-cell types
	Mycosis fungoides and the Sézary syndrome
	Convoluted lymphocyte
	?Immunoblastic sarcoma (of T cells)
	?Hodgkin's disease
III.	B-cell types
	Small lymphocyte (CLL)[a]
	Plasmacytoid lymphocyte
	Follicular center cell (FCC) types (follicular, diffuse, follicular and diffuse, and sclerotic)
	small cleaved[b]
	large cleaved
	small noncleaved[c]
	large noncleaved[d]
	Immunoblastic sarcoma (of B cells)
IV.	Histiocytic type
V.	Unclassifiable

[a] Corresponds to 'well-differentiated lymphocytic' of Rappaport.
[b] Corresponds to 'poorly differentiated lymphocytic' of Rappaport.
[c] Corresponds to 'undifferentiated' of Rappaport.
[d] Corresponds to 'histiocytic' of Rappaport.
From Lukes and Collins [10].

phoid follicle. Furthermore, the malignant cell types within follicular lymphomas were seen as deriving from, or recapitulating, the lymphoid cells within normal germinal centers. The cells within normal lymph node germinal centers were dubbed *follicular center cells* by Lukes and Collins, who divided these cells into small cleaved, large cleaved, small noncleaved, and large noncleaved types, corresponding to proposed normal patterns of lymphocyte differentiation within active germinal centers. Similarly, the Kiel group coined the terms *centrocyte* and *centroblast*, corresponding to the small cleaved and large noncleaved lymphocytes, respectively, of Lukes and Collins.

Ever since immunologic principles were employed in the categorization of malignant lymphoma, subsequent classification schemes have focused upon superimposing specific types of lymphoma onto stages of normal lymphoid ontogeny, or normal stages of antigen-dependent lymphocyte transformation. For instance, mantle cell lymphoma is so named since the cytology, histologic distribution, and immunophenotype of the malignant cells in this lesion resemble those of the mantle zone lymphocytes of normal secondary lymphoid follicles.

The use of presumed normal counterparts in the classification of malignant lymphoma is advantageous, since the similarity of the neoplastic cells to a known normal cell type provides insight into the biology and therefore the potential clinical course of a particular lymphoma. It is important to stress, however, that the biologic traits of a neoplastic cell type rarely exactly recapitulate those of its normal counterpart. In the end, the determination of the biologic potential of a given lymphoma remains an empirical process. At the

very least, however, the ontogenic approach to lymphoma classification allows for the appropriate grouping of biologically comparable lesions.

Translating among classification schemes — the Working Formulation

In 1982, the National Cancer Institute sponsored a study of non-Hodgkin's lymphoma classification, resulting in a Working Formulation of the Non-Hodgkin's Lymphomas for Clinical Usage [12]. Although it was originally proposed only as a means for translating terminology among existing classification schemes, the Working Formulation (WF) has since been adopted as the most commonly used lymphoma classification in the United States (Table 3). The Working Formulation combined the features of existing morphologic and immunologic classification schemes into a single system and further stratified lymphoma categories into three grades based upon the clinical course of over 1150 patients. Despite the incorporation of data from immunologic studies of lymphoma, the Working Formulation remained a purely morphologic classification scheme; no immunophenotypic or genotypic data were included as diagnostic criteria. As a result, a single category may contain more than one distinct disease entity, and, conversely, a single disease may potentially be classified into one of several categories. The Working Formulation did not formally distinguish between B-cell lymphoma and T-cell lymphoma, although the morphologic entities included in the Working Formulation corresponded mainly to B-cell lymphomas in the Kiel and Lukes–Collins classifications. The terminology selected in the Working Formulation resembled the classification of Lukes and Collins (Tables 2 and 3).

The Working Formulation provided a reproducible, and at the time relatively up-to-date, lymphoma classification. Its clinical relevance was further demonstrated in a long-term clinical follow-up study of the original cohort [13]. Indeed, the Working Formulation remains the basis for a large majority of clinical lymphoma trials in the United States. However, advances in lymphoma therapy have to some degree obscured the boundaries between the clinical grades, particularly between the intermediate and high-grade categories [13–16]. This illustrates the pitfalls of organizing a classification around prognostic strata that depend in large part on ever-changing therapeutic protocols.

In the decade following the publication of the Working Formulation, several discrete forms of lymphoma were identified based upon morphologic, immunophenotypic, genetic, and clinical features (see below). Although many of these diseases could be forced into existing Working Formulation morphologic categories, the documentation of specific biological features, as well as characteristic responses to existing and emerging therapies, warranted their recognition as separate clinicopathologic entities. For these reasons, many consider the Working Formulation in its current form to be obsolete.

Table 3. The Working Formulation of the Non-Hodgkin's Lymphomas for Clinical Usage (1982)

Working Formulation category	Rappaport equivalent
Low grade	
A. Malignant lymphoma, small lymphocytic Consistent with CLL Plasmacytoid	Well-differentiated lymphocytic lymphoma
B. Malignant lymphoma, follicular Predominantly small cleaved cell Diffuse areas Sclerosis	Nodular, poorly differentiated lymphocytic lymphoma
C. Malignant lymphoma, follicular Mixed, small cleaved and large cell Diffuse areas Sclerosis	Nodular, mixed lymphocytic/histiocytic lymphoma
Intermediate grade	
D. Malignant lymphoma, follicular Predominantly large cell Diffuse areas Sclerosis	Nodular, histiocytic lymphoma
E. Malignant lymphoma, diffuse Small cleaved cell Sclerosis	Diffuse, poorly differentiated lymphocytic lymphoma
F. Malignant lymphoma, diffuse Mixed, small and large cell Sclerosis Epithelioid cell component	Diffuse, mixed lymphocytic/histiocytic lymphoma
G. Malignant lymphoma, diffuse Large cell Cleaved cell Noncleaved cell Sclerosis	Diffuse histiocytic lymphoma
High grade	
H. Malignant lymphoma Large cell, immunoblastic Plasmacytoid Clear cell Polymorphous Epithelioid cell component	
I. Malignant lymphoma Lymphoblastic Convoluted cell Nonconvoluted cell	
J. Malignant lymphoma Small noncleaved cell Burkitt's Follicular areas	Undifferentiated lymphoma
Miscellaneous	
Composite	
Mycosis fungoides	
Histiocytic	
Extramedullary plasmacytoma	
Unclassifiable	
Other	

From the Non-Hodgkin's Lymphoma Pathologic Classification Project [12].

Current and future lymphoma classifications

Updated Kiel classification

Since its original publication in 1974, the Kiel classification has been updated to accommodate more recently recognized entities [17,18] (Table 4), and the revised Kiel classification maintains widespread usage within Europe. While the original Kiel classification did not formally distinguish B-cell from T-cell lymphomas, the morphologic categories were practically weighted toward B-cell neoplasms. Revision of the Kiel classification focused mainly upon the formal separation of B- and T-cell lymphomas, and upon the further subcategorization of the T-cell lymphomas. The revised Kiel classification was solidly grounded in theoretical models of lymphocyte development. Still, however, immunophenotypic, genotypic, and cytogenetic data (beyond designation of T- and B-cell status) were not formally included within diagnostic criteria.

The revised Kiel classification presented practical advantages over other systems for the diagnosis of certain lymphomas. One study specifically advocated the use of the revised Kiel classification for the subcategorization of small B-cell neoplasms [19], a group of diseases not adequately addressed by the Working Formulation. The utility of revised Kiel in the diagnosis of B-cell lymphomas is underscored by its recognition of mantle cell lymphoma (called *diffuse centrocytic lymphoma*) prior to the formal inclusion of this entity in other systems.

The formal classification of T-cell lymphomas within the revised Kiel classification, based on the proposal of Suchi et al. [20], set it apart from other popular systems, including the Working Formulation. However, critics

Table 4. Updated Kiel classification of non-Hodgkin's lymphoma

B cell	T cell
Low grade	
Lymphocytic — chronic lymphocytic and prolymphocytic leukemia; hairy cell leukemia	Lymphocytic — chronic lymphocytic and prolymphocytic leukemia
Lymphoplasmacytic/lymphoplasmacytoid	Lymphoepithelioid
Plasmacytic	Angioimmunoblastic
Centroblastic/centrocytic	T zone
Centrocytic	Pleomorphic, small cell
High grade	
Centroblastic	Pleomorphic, medium and large cell
Immunoblastic	Immunoblastic
Large cell anaplastic	Large cell anaplastic
Burkitt's lymphoma	
Lymphoblastic	Lymphoblastic
Rare types	Rare types

Adapted from Stansfeld et al. [17] and Lennert and Feller [18].

stressed that the categories of T-cell lymphoma within the revised Kiel system suffered from poor interobserver reliability and were not necessarily useful in clinical practice [21,22].

The revised Kiel classification improved upon existing classification schemes, but it also relied heavily upon the purely morphologic subclassification of lymphomas, without necessary regard to the clinical importance (or lack of clinical importance) of such categorization. To some investigators, this approach only furthered the stereotype of lymphoma classifications as being unnecessarily complex and esoteric [4].

REAL (ILSG) classification

In 1994, the International Lymphoma Study Group (ILSG), consisting of several hematopathologists from the U.S. and Europe, proposed a new classification of lymphoid neoplasms, which they named the Revised European–American Lymphoma (REAL) classification [23–25] (Table 5). The ILSG's stated purpose was to clarify some of the confusion surrounding lymphoma classification, to provide an updated classification that would include newly recognized disease categories, to address issues of interobserver reliability, and to bring lymphoma classification more in line with the current state of practice in hematopathology. Although prior classifications separately recognized B-cell and T-cell lymphomas, the REAL classification was the first to formally incorporate immunophenotypic, genotypic, and cytogenetic characteristics in defining specific disease entities. Furthermore, the REAL classification took the long-overdue step of defining true clinicopathologic disease entities, rather than simply relying heavily upon morphology or patterns of lymphocyte differentiation. The definitions of certain lymphomas in the REAL classification included anatomic sites of occurrence (when relevant), etiologic agents (when relevant), and the demographics of specific lymphomas. The recognition that discrete diseases are defined by multiple clinical and laboratory parameters, and not simply by histology, was a significant step forward in lymphoma classification.

The REAL classification laid the groundwork for a modern, multiparameter approach to lymphoma classification. However, its publication met with considerable controversy. Some felt that the ILSG did not go far enough in designing the REAL classification around the clinical relevance of specific lymphomas [5]. At the opposite extreme, others criticized the inclusion of any clinical and immunophenotypic criteria and suggested that lymphoma classification should rely solely upon light microscopic examination [26]. The REAL classification met with further controversy regarding what was perceived as the inappropriate 'splitting' of certain disease categories and the inappropriate 'lumping' of others. For instance, the category of immunoblastic lymphoma, recognized in prior classifications, was not recognized by REAL as distinct from other large B-cell lymphomas. The REAL classification did recognize,

Table 5. Non-Hodgkin's lymphoid neoplasms recognized by the Revised European–American Lymphoma (REAL) classification

B-cell neoplasms

I. Precursor B-cell neoplasm: precursor B-lymphoblastic leukemia/lymphoma
II. Peripheral B-cell neoplasms
  1. B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma
  2. Lymphoplasmacytoid lymphoma/immunocytoma
  3. Mantle cell lymphoma
  4. Follicle center lymphoma, follicular
     Provisional cytologic grades: I (small cell), II (mixed small and large cell), III (large cell)
     Provisional subtype: diffuse, predominantly small cell type
  5. Marginal zone B-cell lymphoma
     Extranodal (MALT-type +/− monocytoid B cells)
     Provisional subtype: nodal (+/− monocytoid B cells)
  6. Provisional entity: splenic marginal zone lymphoma (+/− villous lymphocytes)
  7. Hairy cell leukemia
  8. Plasmacytoma/plasma cell myeloma
  9. Diffuse large B-cell lymphoma[a]
     Subtype: primary mediastinal (thymic) B-cell lymphoma
  10. Burkitt's lymphoma
  11. Provisional entity: high-grade B-cell lymphoma, Burkitt's-like[a]

T-cell and putative NK-cell neoplasms

I. Precursor T-cell neoplasm; precursor T-lymphoblastic lymphoma/leukemia
II. Peripheral T-cell and NK-cell neoplasms
  1. T-cell chronic lymphocytic leukemia/prolymphocytic leukemia
  2. Large granular lymphocyte leukemia (LGL)
     T-cell type
     NK-cell type
  3. Mycosis fungoides/Sezary syndrome
  4. Peripheral T-cell lymphomas, unspecified[a]
     Provisional cytologic categories: medium-sized cell, mixed medium and large cell, large cell, lymphoepithelioid cell
     Provisional subtype: Hepatosplenic γδ T-cell lymphoma
     Provisional subtype: Subcutaneous panniculitic T-cell lymphoma
  5. Angioimmunoblastic T-cell lymphoma (AILD)
  6. Angiocentric lymphoma
  7. Intestinal T-cell lymphoma (+/− enteropathy associated)
  8. Adult T-cell lymphoma/leukemia (ATL/L)
  9. Anaplastic large cell lymphoma (ALCL), CD30+, T- and null-cell types
  10. Provisional entity: anaplastic large-cell lymphoma, Hodgkin's-like

[a] Categories considered likely to include more than one disease entity.
Adapted from Harris et al. [23].

however, that the broad category of large B-cell lymphoma likely included more than one distinct disease entity, and that concerns over interobserver reliability warranted the grouping of previously distinct entities.

As with other classification schemes, the publication of the REAL classification was followed by assessments of its clinical relevance and reliability [16,27–33]. In the largest of these studies, over 1400 cases of non-Hodgkin's lymphoma were reviewed according to ILSG criteria [16]. The REAL classification was found to be a readily applicable, clinically relevant, and reproduc-

ible scheme for lymphoma diagnosis. In particular, the authors of this study found the inclusion of immunophenotypic data as diagnostic criteria to be clinically useful. However, they stressed that the clinical behavior may be widely variable within a single disease entity and that, for appropriate clinical decision making, prognosis was best determined by considering histologic classification of a given lymphoma in conjunction with prognostic factors as defined by the International Prognostic Index [34].

Revised WHO classification

Even as the REAL classification was being devised, plans were under way for the development of a World Health Organization (WHO)-sponsored classification of hematologic neoplasms. At the time of this writing, the WHO classification is not yet complete. However, drafts of the evolving document have been circulated [35]. The working document bears much resemblance to the REAL classification. However, the WHO classification extends itself to include additional recently recognized entities, while maintaining the contemporary approach advocated by the ILSG in the REAL publication. For instance, the posttransplantation lymphoproliferative disorders and other immunodeficiency-related neoplasms were not directly addressed by the ILSG, but are included in the proposed WHO classification.

An overview of newly emerging lymphoma entities

An exhaustive survey of specific lymphoma entities is available in many excellent works and is beyond the scope of this review [9,23,24,35]. However, with the evolution of new lymphoma classification systems based upon a combination of morphologic, immunophenotypic, genetic, and clinical features, several apparently distinct disease entities have emerged since the publication of the Working Formulation. Following is a selected sampling of lymphoma entities recognized in emerging classification schemes. These entities are highlighted because they demonstrate the integrated use of morphologic, immunophenotypic, genetic, and clinical data in the subclassification of lymphoma.

Mantle cell lymphoma

Although the disease currently known as mantle cell lymphoma has been recognized for many years, the distinct nature of this neoplasm has been appreciated only relatively recently.

Over 25 years ago, there were reports of non-Hodgkin's lymphoma with cytologic features intermediate between small lymphocytic lymphoma (*well differentiated lymphocytic lymphoma* by Rappaport) and follicular small cleaved cell lymphoma (*poorly differentiated lymphocytic lymphoma* by

Rappaport) [36]. These tumors were thus designated as *intermediately differentiated lymphocytic lymphoma* (IDLL), and were thought by some investigators to represent the neoplastic counterpart to cells of the primary lymphoid follicle or of the mantle zone of the secondary lymphoid follicle [37]. This type of lymphoma was identical to the diffuse centrocytic lymphoma of the Kiel classification [11,18]. Although not easily accommodated by the Working Formulation, these tumors could conceivably be classified into one of several categories, depending on the histologic features (e.g., small lymphocytic or diffuse small cleaved). By the early 1990s, the term *mantle cell lymphoma* had been accepted to describe these lymphomas [38], although no major classification system listed mantle cell lymphoma until the REAL classification was published in 1994 [23,24].

Mantle cell lymphoma is distinct histologically, immunophenotypically, clinically, and genetically. Histologically, three major architectural patterns have been described in mantle cell lymphoma: diffuse (diffuse effacement of lymph node architecture), nodular (formation of discrete nodules occupied by lymphoma cells), and mantle zone (neoplastic expansion of mantle zones surrounding reactive germinal centers) [39,40] (Figure 1). Several studies have yielded conflicting data regarding the relevance of histologic architecture to clinical outcome. Some investigators have demonstrated significantly prolonged survival in cases demonstrating a mantle-zone or nodular pattern of

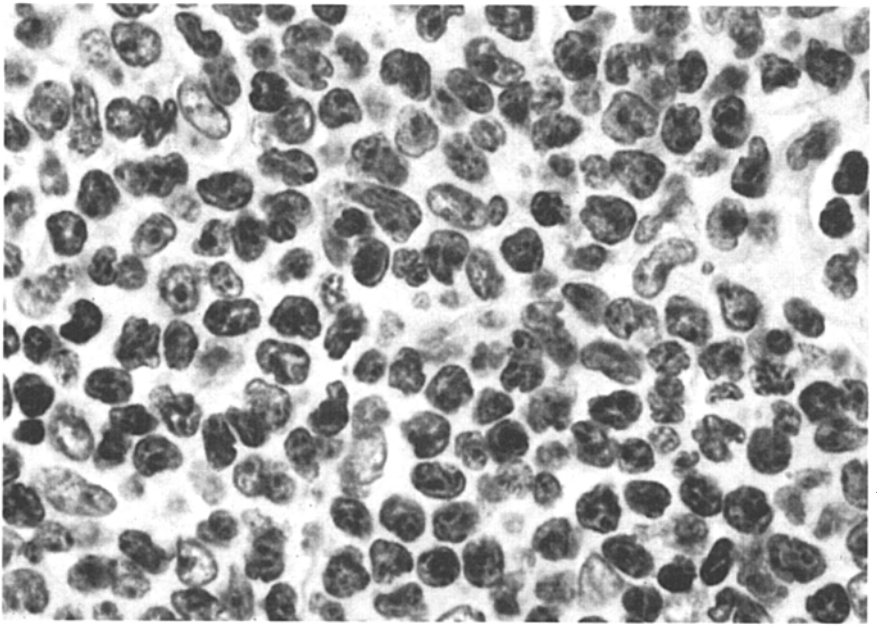

Figure 1. Mantle cell lymphoma. There is a monotonous proliferation of small, irregular cells with course chromatin and scant cytoplasm.

infiltration [28,41,42], while others have shown similar outcomes regardless of histologic subtype [40,43,44].

The immunophenotype of mantle cell lymphoma is distinct. Like B-cell chronic lymphocytic leukemia (CLL), mantle cell lymphoma is a B-cell lymphoid neoplasm that co-expresses the T-cell-associated antigen CD5. In contrast to CLL, however, mantle cell lymphoma generally does not express the activated B-cell antigen CD23 but does express the resting B-cell marker FMC7 [45,46]. The lymphocytes of mantle cell lymphoma generally express surface IgM or IgD, and the surface density of immunoglobulin per cell is generally greater in mantle cell lymphoma than in CLL [45].

Mantle cell lymphoma has been linked to a distinct translocation between the long arms of chromosome 11 and 14, t(11;14)(q13;q32) [47]. On the molecular level, this translocation juxtaposes the joining region of the immunoglobulin heavy chain gene on chromosome 14 to a region adjacent to the BCL-1 gene (also known as PRAD-1 or CCND-1) [48]. The t(11;14) can be detected by cytogenetic or molecular genetic methods in the majority of mantle cell lymphoma cases. The true incidence of the t(11;14) in mantle cell lymphoma is difficult to assess, since cytogenetic studies are difficult to carry out in mature lymphocytic neoplasms, and several molecular breakpoints spanning a large distance have been identified near the BCL-1 gene. The BCL-1 gene encodes for the cyclin-D1 protein, which is overexpressed in a large majority of mantle cell lymphoma. Cyclin D1 overexpression may be of help diagnostically, since it is observed very rarely in other B-cell neoplasms [49,50].

When mantle cell lymphoma was first recognized as a distinct entity, its morphologic similarity to low-grade lymphomas led to assumptions that it would be associated with a low-grade, protracted clinical course. In fact, mantle cell lymphoma is an aggressive disease that responds poorly to conventional and emerging therapies and has one of the worst prognoses of all non-Hodgkin's lymphomas [16]. Weisenberg and Armitage [39] have noted that this disease '... brings together the worst characteristics of high-grade and low-grade lymphomas.' Like high-grade lymphomas, mantle cell lymphoma is aggressive with a median survival in the range of 3 to 5 years [39,40]. However, like many low-grade lymphomas, it is usually disseminated at presentation [40] and is considered incurable by current therapeutic methods [38].

Marginal zone lymphoma

In the 1980s, there were descriptions of B-cell lymphomas marked by the proliferation of morphologically distinct cells termed *monocytoid B cells* [51,52]. The cells of monocytoid B-cell lymphoma were morphologically identical to the reactive monocytoid or parafollicular B cells seen in various reactive states, particularly toxoplasmic lymphadenitis. Based on morphologic and phenotypic similarities, it was suggested that these cells were related to lymphocytes of the splenic marginal zone — a histologically distinct sheath of

lymphocytes normally found surrounding the lymphoid follicles of the normal spleen [53,54]. During the same general time period, B-cell lymphomas arising in mucosa-associated lymphoid tissue (MALT) were also described in which the neoplastic cells bore cytologic, histologic, and phenotypic similarities to marginal zone lymphocytes [55]. In the REAL classification, the category of marginal zone B-cell lymphoma is divided into three main categories: low-grade B-cell lymphoma of MALT, primary splenic marginal zone lymphoma, and node-based marginal zone lymphoma (including monocytoid B-cell lymphoma); the last two are currently provisional categories.

Low-grade lymphoma of mucosa-associated lymphoid tissue (MALT). Low-grade MALT lymphomas have most commonly been described in the gastrointestinal tract, salivary gland, lung, and lacrimal gland [55–61]. In addition, lymphomas of MALT type have been described in a large number of other anatomic sites, including thyroid gland, skin, kidney, uterine cervix, breast, bladder, liver, and even dura [62–66]. Neither the Working Formulation nor the Kiel classification directly address this lymphoma type; these cases would be classified in the Working Formulation (or Kiel classification) as either small lymphocytic (lymphocytic) or small lymphocytic plasmacytoid (lymphoplasmacytoid/lymphoplasmacytic). The distinct nature of this entity is underscored by its different clinical presentation, behavior, and response to therapy compared to other low-grade B-cell lymphomas.

MALT lymphomas at various sites generally display similar morphologic features. There is usually a proliferation of small, mature-appearing lymphocytes with slightly irregular nuclei and scant to moderate amounts of pale cytoplasm (centrocyte-like or marginal zone cells); variable numbers of monocytoid B cells may be seen as well. The lymphomatous infiltrate typically invades adjacent epithelial or glandular structures, forming the characteristic lymphoepithelial lesion of low-grade MALT lymphoma [55] (Figure 2). The small neoplastic lymphocytes can also be seen infiltrating individually or in small clusters into bystander reactive follicles in a process termed *follicular colonization* [67]. Plasma cell differentiation is also a common feature [55].

In general, low-grade MALT lymphomas tend to be localized and follow an indolent and relatively benign clinical course [55], although clinical behavior may be site dependent [68]. Five-year survival rates of over 80% are cited for low-grade gastric MALT lymphoma [69,70]. With the exception of some gastric MALT lymphomas (see below), therapy is often directed at local control, either by excision or irradiation. Relapses tend to be at the primary site of involvement, although late recurrence at remote MALT sites is being reported with increasing frequency.

In the stomach, low-grade MALT lymphoma has been linked with *Helicobacter pylori* infection. In fact, gastric low-grade MALT lymphoma may completely regress with antimicrobial therapy directed at eradicating *H. pylori*

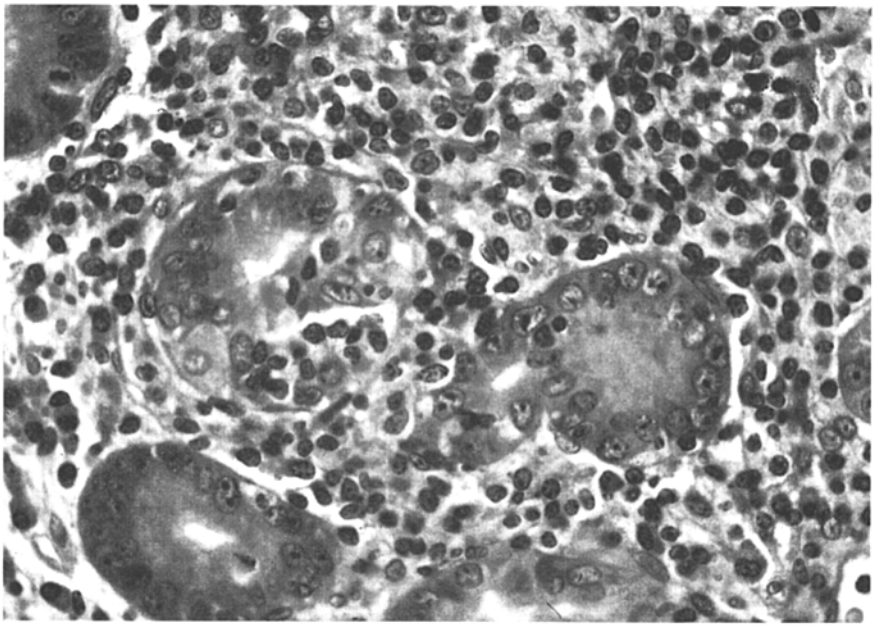

Figure 2. A characteristic lymphoepithelial lesion of low-grade lymphoma of mucosa-associated lymphoid tissue (MALT). Small irregular cells are seen infiltrating and destroying a gastric gland.

[69,71]. This outcome has led to debate regarding the true neoplastic nature of low-grade MALT lymphomas of the stomach. However, demonstration of clonal immunoglobulin gene rearrangements and clonal cytogenetic abnormalities, as well as the occasional spread of gastric MALT lymphoma beyond the primary site of involvement, has led to general acceptance of these lesions as low-grade lymphomas.

Occasionally, low-grade MALT lymphoma may transform to a diffuse large B-cell lymphoma [70]. This phenomenon may be difficult to distinguish from de novo large cell lymphoma occurring at a MALT site, but the distinction may be clinically relevant. A recent study has documented improved survival in gastric large cell lymphoma containing either foci of more typical low-grade MALT lymphoma or lymphoepithelial lesions composed of large cells, when compared to pure large cell lymphoma [70]. The authors of this study suggest using the term *high-grade MALT* for large cell lymphomas with such features in order to convey this difference in prognosis.

No consistent genetic abnormality has been described in low-grade MALT lymphomas. However, trisomy 3 has been documented in many of these tumors [72]. More recently, the translocation t(11;18)(q21;q21) has been described in several cases [73]. Further study will be necessary to characterize this abnormality on the molecular genetic level.

Node-based marginal zone lymphoma (monocytoid B-cell lymphoma). Primary lymph node-based marginal zone B-cell lymphoma is recognized as a provisional entity in the REAL classification (Table 5). The basis for its provisional status is the observation that many lymph nodes involved by marginal zone lymphoma occur in patients with low-grade MALT lymphomas at extranodal sites or in patients with SMZL [23,74] (Figure 3). This finding has led some to believe that true node-based marginal zone lymphoma is at least rare and at most nonexistent as a distinct disease [23]. However, recent data suggest that a minority of nodal marginal zone lymphomas do represent a distinct entity [31,75]. Based on a limited number of cases, these lymphomas appear to present more like traditional types of low-grade B-cell lymphoma with peripheral adenopathy and disseminated disease. They also show shorter failure-free survival than extranodal marginal zone lymphomas [31].

Splenic marginal zone lymphoma. Recently a type of low-grade B-cell lymphoma arising primarily in the perifollicular marginal zone of the spleen has been recognized as a distinct clinicopathologic entity [74,76,77] (Figure 4). Splenic marginal zone lymphoma tends to affect middle-aged or older individuals, with median onset in the seventh decade. Presenting signs and symptoms vary, but many patients present initially with abdominal pain, weight

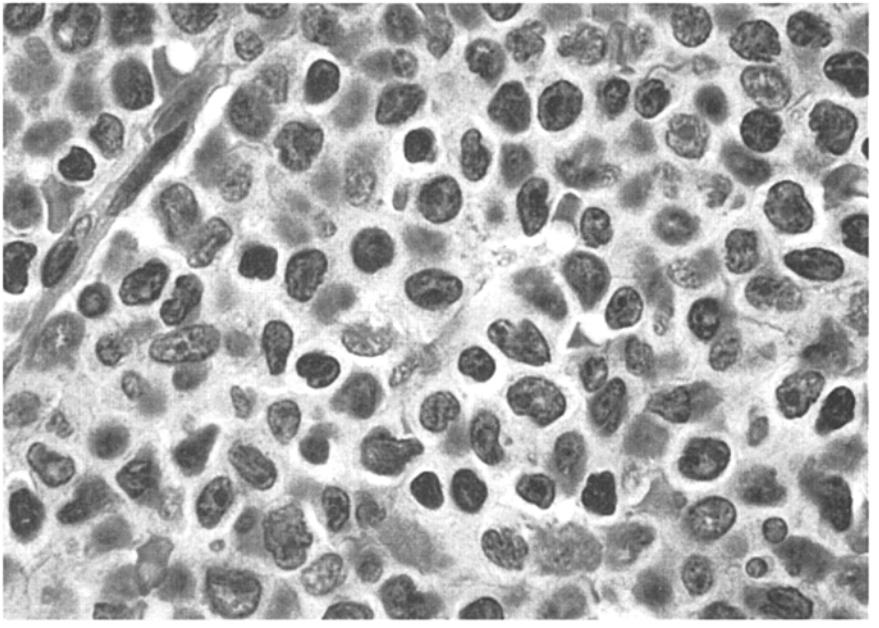

Figure 3. Node-based marginal zone B-cell lymphoma (monocytoid B-cell lymphoma). The cells are medium-sized with elongate, bent, and angulated nuclei, more open chromatin than a mature lymphocyte, variably prominent nucleoli, and abundant pale cytoplasm.

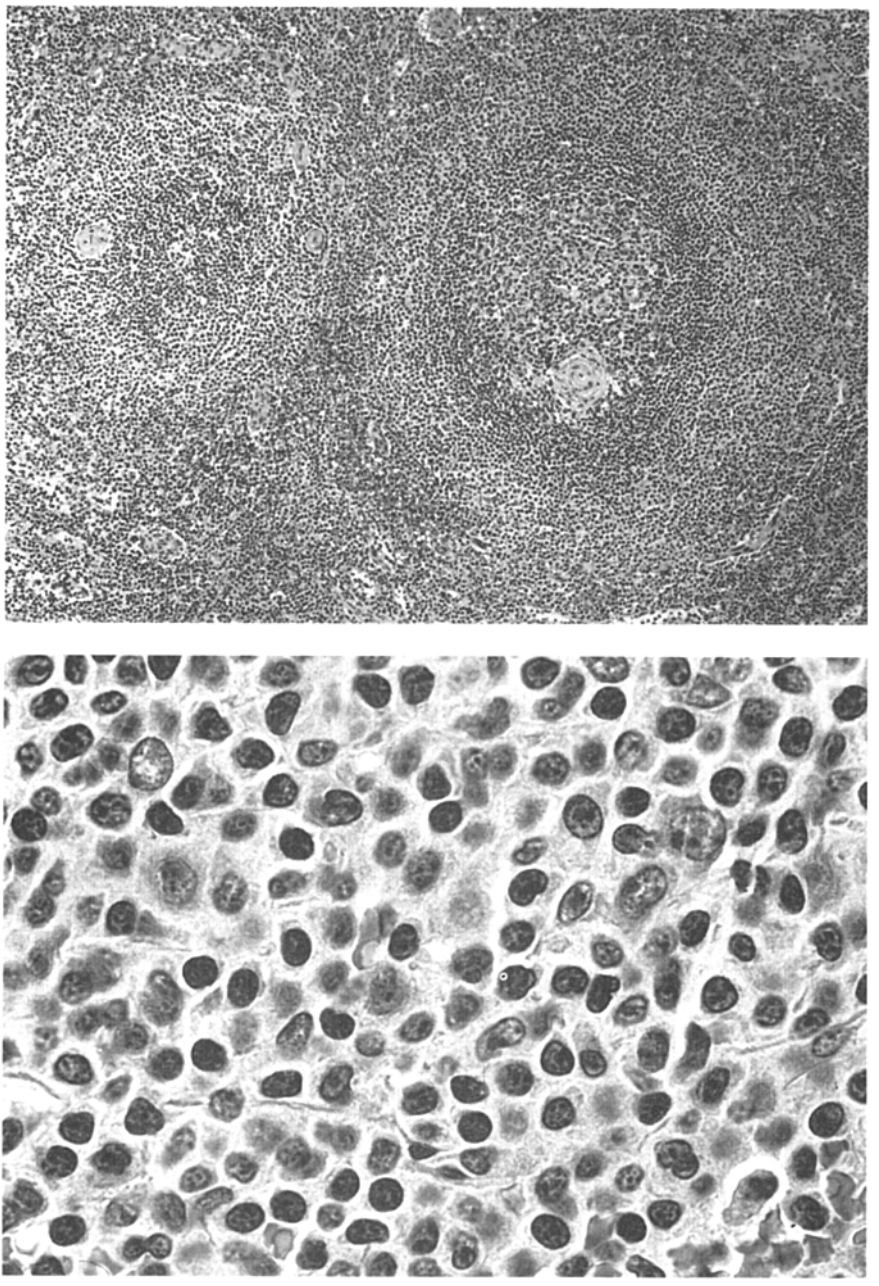

Figure 4. Splenic marginal zone lymphoma. **A.** Medium-power photomicrograph showing two splenic white pulp follicles with distinct peripheral pale zones formed by neoplastic marginal zone lymphocytes. **B.** High power shows characteristic marginal zone cells. These are similar to those in Figure 3 except that they have smaller, less irregular nuceli, more condensed chromatin, and less cytoplasm.

loss, and peripheral blood cytopenias. Occasionally these are incidentally detected following spontaneous or traumatic splenic rupture [78,79].

The neoplastic infiltrate of SMZL shows a remarkable resemblance to the normal splenic marginal zone [74,76,77]. The pattern of infiltration shows a striking marginal zone pattern (a sheath of neoplastic cells external to variably preserved germinal centers and mantle zones), and cytologically the cells are identical to their normal counterparts. The consituent cells also closely resemble those of nodal and extranodal marginal zone lymphomas.

Most patients with splenic marginal zone lymphoma present with stage III or IV disease [74,77]; however, the bulk of disease is present in the spleen, and peripheral adenopathy is distinctly uncommon. Despite disseminated disease at presentation, the clinical course is typically indolent, and prolonged survival, or even complete clinical and hematological remission, has been reported in response to splenectomy alone [74,80].

Leukemic involvement by splenic marginal zone lymphoma is not uncommon; approximately half of cases show an absolute lymphocytosis, and roughly 30% more have atypical lymphocytes detectable in the blood without lymphocytosis [74,76,77]. In fact, the disease entity previously known as splenic lymphoma with circulating villous lymphocytes (SLVL) is now thought by most investigators to represent a clinical variant of SMZL [81]; approximately one quarter of SMZL cases present with SLVL features [74,76,77]. Furthermore, many cases previously diagnosed as B-cell chronic lymphocytic leukemia, or as *atypical CLL*, may represent the leukemic phase of splenic marginal zone lymphoma [82,83].

Anaplastic large cell lymphoma

Anaplastic large cell lymphoma (ALCL) is a histologically distinct neoplasm marked by the infiltration of large, bizarre-appearing cells (Figure 5). Typically, these cells include a mixture of multinucleated giant cells and cells with curved, wreath-like peripheral nuclei. The pleomorphic cells of ALCL can closely resemble the Reed–Sternberg cells of Hodgkin's disease, and it is likely that many or most of the lymphomas previously classified as lymphocyte-depleted Hodgkin's disease actually represent ALCL. ALCL was originally distinguished as a non-Hodgkin's lymphoma that consistently expressed the activation marker Ki-1 (CD30) [84]. Thus, the term *Ki-1 lymphoma* was used after the initial descriptions of this disease.

ALCL has a propensity to involve extranodal sites. If untreated, ALCL follows a very aggressive clinical course. However, with appropriate multiagent chemotherapy, these tumors appear to have one of the most favorable prognoses among the non-Hodgkin's lymphomas, with disease-free survival rates in the range of 50%–60% at five years [16,85–87], and overall five-year survival in excess of 70% [16]. A distinct clinical variant of ALCL involves only the skin at presentation. Primary cutaneous ALCL without extracutaneous involvement carries a distinctly favorable prognosis, with re-

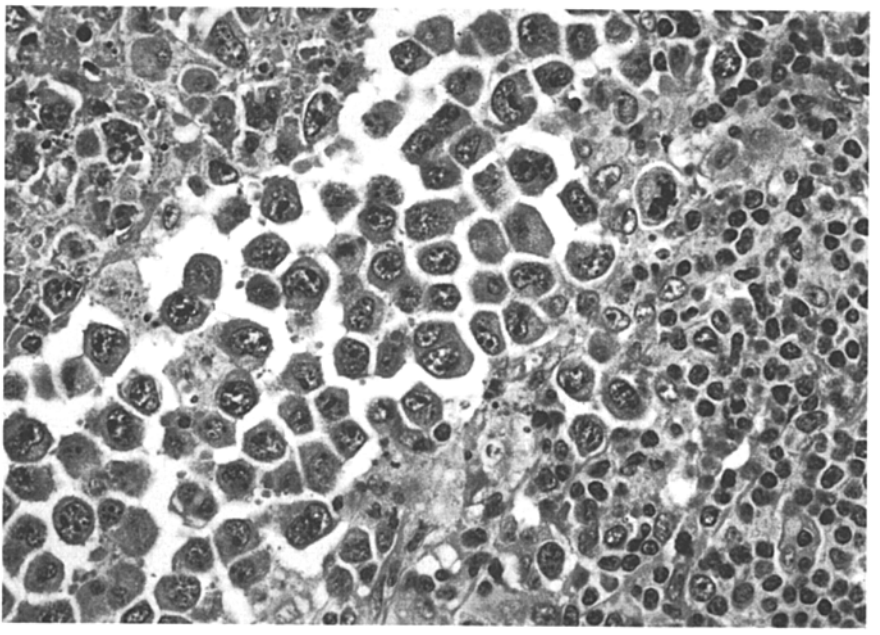

Figure 5. Anaplastic large cell lymphoma (ALCL). Sinusoidal infiltrate of atypical cells with abundant cytoplasm and prominent nucleoli.

ports of spontaneous regression in some cases [85,88]. In fact, these cases appear to be more closely related to the benign skin condition lymphomatoid papulosis than to systemic ALCL. A four-year survival rate of 92% was detected in one study [88].

The neoplastic cells of ALCL tend to mark either as T lymphocytes or as 'null' cells void of T- or B-antigen expression. B-cell ALCL is well described, but recent evidence supports the notion that B-cell ALCL is a biologically distinct lesion from T or null ALCL and does not differ significantly from other diffuse large B-cell lymphomas [29,89]. Therefore, many investigators recommend excluding B-cell neoplasms from the ALCL category, although this remains controversial [90]. The REAL classification formally recognizes only the T-cell and null-cell ALCL types [23], whereas the updated Kiel classification recognizes B-cell and T-cell types [17]. ALCL is not specifically recognized in the Working Formulation and would most likely be classified as large cell immunoblastic lymphoma, pleomorphic subtype.

CD30-positive anaplastic large cell lymphoma (ALCL) of the T-cell or null-cell type is strongly linked to the presence of the chromosome translocation t(2;5)(p23;q35) within the malignant cells [91–93]. The general absence of the t(2;5) in B-cell ALCL adds additional support to the exclusion of B-cell tumors from this category. The t(2;5) corresponds to the fusion of the ALK (anaplastic lymphoma kinase) and NPM (nucleophosmin) genes at the molecular level,

and it is thought to play a key role in the biologic development of ALCL [94]. Recent evidence has supported a shift toward considering genetic alteration of the ALK locus at chromosome 2p23 as the defining feature of ALCL [29,93,95,96]. Expression of the ALK protein has also been closely linked to ALCL and the t(2;5), making immunohistochemical detection of ALK a potentially useful tool in the differential diagnosis of large cell lymphomas [95–98].

Posttransplantation lymphoproliferative disorders (PT-LPD)

The separate categorization of immunodeficiency-related lymphoproliferative disorders has not been formally recognized in major lymphoma classifications. However, with recent advances in organ transplantation and the increase in AIDS-related neoplasia, immunodeficiency-related lymphoid proliferations have achieved more widespread recognition. Early drafts of the upcoming revised WHO classification indicate that this classification will contain a section on immunodeficiency-related processes, including lymphoproliferative disorders related to iatrogenic immunosupression following organ transplantation.

Posttransplantation lymphoproliferative disorders (PT-LPDs) are seen with greater frequency with the advent of more potent immunosuppressive medications. The rate of PT-LPD varies widely in different studies and with different immunosuppression regimens. The highest rates of occurrence have been in cardiac transplant patients, with ranges between 1% and 13% in some series [99].

With occasional notable exceptions, PT-LPDs are generally B-cell proliferations driven by Epstein–Barr virus infection in the immunocompromised host [100–102]. Clinical, histologic, and molecular genetic studies have demonstrated that PT-LPDs form a wide and continuous spectrum of disease, ranging from completely reversible, infectious mononucleosis-like processes to aggressive, high-grade lymphomas [100,102–108]. Since these diseases form a continuum from the reactive to the neoplastic, they are difficult to place into discrete categories using existing lymphoma classifications.

As a practical matter, therapy for PT-LPDs is based upon the likelihood that the lesion will regress solely in response to the reduction or withdrawal of immunosuppressive medication or upon whether conventional chemotherapy is needed [107,109]. The early plasma cell hyperplasias reliably respond to the withdrawal of immunosuppression [110]. In contrast, the histologically monomorphic or high-grade lesions usually progress if they are not actively treated. The most difficult area to effectively classify falls within the middle of the spectrum, within the so-called polymorphic PT-LPDs (Figure 6).

Several studies have focused upon distinguishing the line beyond which a polymorphic PT-LPD becomes a clinical lymphoma. A histologic distinction has been made between *polymorphic B-cell hyperplasia*, which demonstrates a heterogeneous mixture of lymphocytes, evidence of plasma cell differentia-

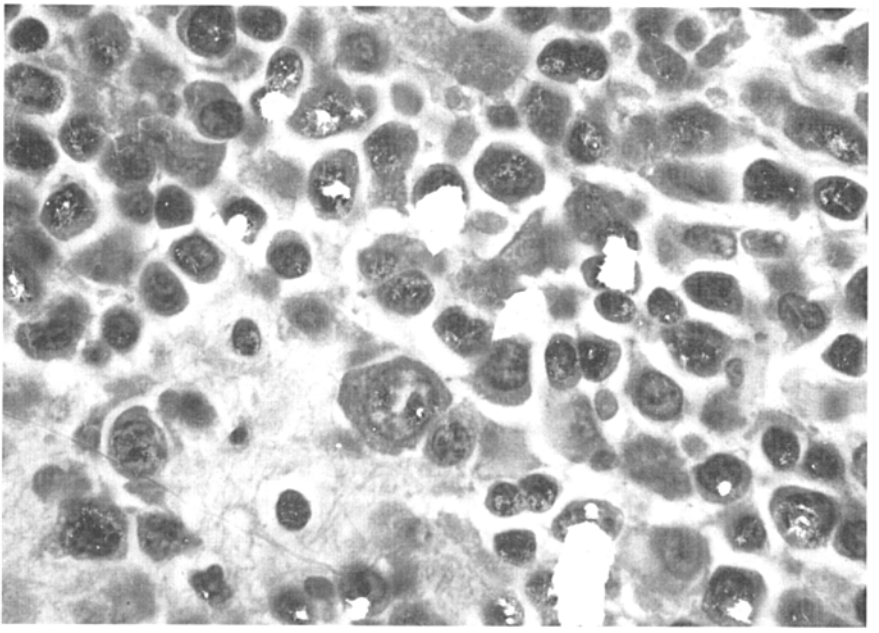

Figure 6. Polymorphic posttransplantation lymphoproliferative disorder. There is a spectrum of lymphocyte morphology, including small lymphocytes, plasmacytoid lymphocytes, and immunoblasts.

tion, and a lack of significant cytologic atypia or geographic necrosis, and *polymorphic B-cell lymphoma*, which demonstrates more overt cytologic atypia and larger regions of necrosis [104]. Unfortunately, this histologic distinction does not effectively predict outcome. Many polymorphic B-cell lymphomas regress in response to decreased immunosuppression. Molecular clonality studies have been similarly unsuccessful in predicting regression versus progression in the polymorphic PT-LPDs [107]. Evidence of clonal Epstein–Barr virus integration or monoclonal B-cell proliferation is well documented in lesions that regress without chemotherapeutic intervention. Likewise, polyclonal proliferations can be clinically aggressive. Due to the unpredictable behavior of these PT-LPDs, newer proposals do not formally distinguish between polymorphic B-cell hyperplasia and polymorphic B-cell lymphoma. These terms have been largely replaced by the broader category of *polymorphic PT-LPD* [102]. Depending upon the clinical setting, first-line therapy of polymorphic PT-LPDs (regardless of B-cell clonality) tends to include a trial of either decreasing or withdrawing immunosuppressive medications.

Some types of PT-LPD present clinically and histologically as overt lymphoma. These lesions tend to display diffuse large cell or immunoblastic morphology. Molecular analysis has demonstrated a greater tendency toward

rearrangement of c-myc or other oncogenes within these tumors [102]. These lesions represent the monomorphic PT-LPDs, and they are often diagnosed and treated according to existing non-Hodgkin's lymphoma classification schemes.

The future of lymphoma classification

As our basic knowledge of the immune system continues to advance, there is ever-increasing opportunity to develop lymphoma classifications that provide unprecedented scientific accuracy and clinical relevance. It is clear that approaches to classification that rely solely upon characteristic histologic patterns are obsolete. The mission of current and future lymphoma classifications will be to define relevant clinicopathologic entities, based not only on histomorphology but also upon immunophenotype, genotype, etiology (viral, environmental, genetic), epidemiology, demographic distribution, anatomic site of occurrence, and response to existing and emerging therapies. The temptation to identify interesting but functionally irrelevant morphologic variants must give way to the vision of defining truly distinct and medically relevant diseases. Finally, lymphoma classifications should be continually reexamined and must maintain the flexibility to accommodate new developments in our understanding of hematopoietic neoplasia.

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2. Epstein–Barr virus and lymphoma

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Epstein–Barr virus has been associated with a variety of malignancies. Originally discovered in African Burkitt's lymphoma, it is also found in lymphoproliferative disease arising in immunocompromised populations, in Hodgkin's disease, and in peripheral T-cell lymphoma. The association is of interest in terms of the pathogenesis of these tumors and is becoming important in terms of new approaches to treatment. The purpose of this chapter is to review aspects of the biology of the virus that are relevant to tumorigenesis, review the nature of the association with each of the tumors, and to consider therapeutic implications where these have been defined.

Aspects of viral biology

The EBV genome is approximately 171 kb of double-stranded DNA [1]. It encodes approximately 80 open reading frames. Infection may be lytic or latent. In lytic infection, virions are produced. Virion production requires expression of a variety of viral enzymes for synthesis of viral DNA and synthesis of structural proteins that will form the virus capsid [2]. In latent infection, there is no production of new virions. Viral DNA is replicated by host cell enzymes. However, the virus is not silent in latency. To the contrary, it can exercise a profound influence over the growth properties of infected cells. Indeed an important difference between EBV and other human herpes viruses such as herpes simplex and varicella-zoster virus is that whereas these other viruses are associated with disease only during the lytic part of their life cycle, EBV is also associated with disease during the latent part of the life cycle.

EBV, like most other human herpes viruses, is widespread. Serologic studies show that in all racial, ethnic, and geographic groups, the majority of the world's adult population is infected by the virus [3]. Primary infection in young adulthood or later life is associated with the syndrome of infectious mononucleosis approximately one third of the time [4]. In contrast, primary infection in childhood is almost always asymptomatic. Symptomatic or asymptomatic, infection is common, but associated malignancies are rare. In

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this regard, EBV is like other human viruses associated with tumors. HTLVI, hepatitis B, and papillomavirus-associated tumors only develop in a small fraction of those infected. EBV differs from these tumor viruses in that infection is much more common.

Latent and lytic infection

Initial infection of B cells probably occurs in the oral mucosa following salivary transmission (Figure 1) [3,5]. These latently infected B cells are driven to proliferate, and the pool of infected cells expands without the requirement for production of new virions [6]. Early in infection, as many as several percent of lymphocytes may harbor virus [7–9].

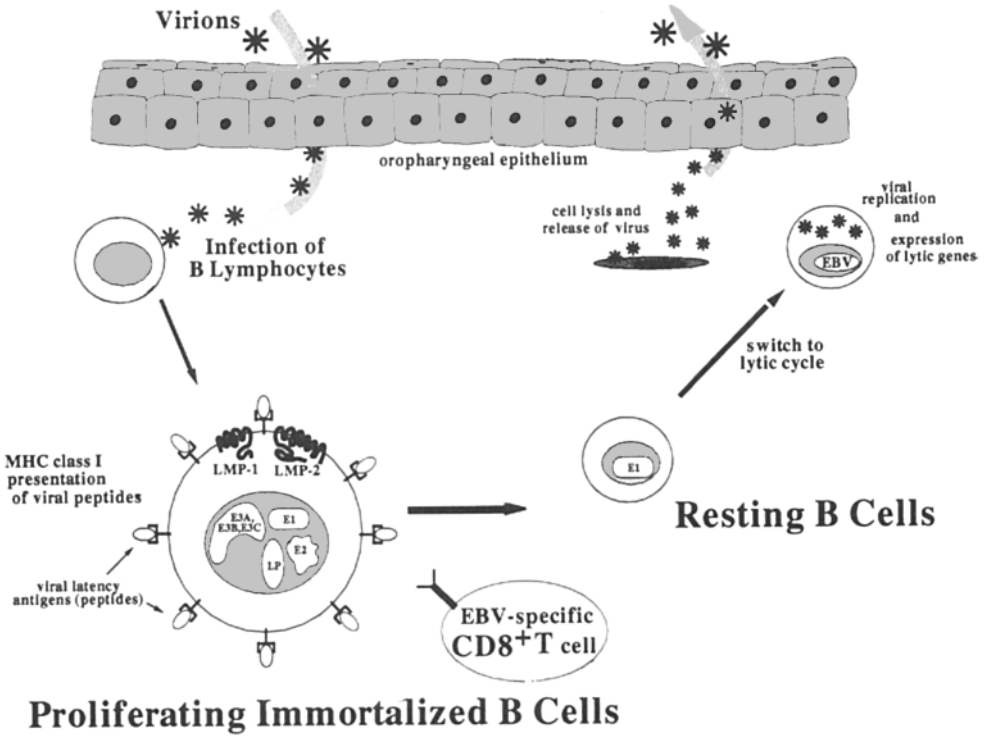

Figure 1. The EBV life cycle. Infection is transmitted in saliva. Lymphocytes of the oropharyngeal epithelium are infected. These B cells proliferate and spread through the B-cell compartment. Eventually a virus-specific T-cell response controls this proliferation. Resting B cells with limited antigen expression persist. These constitute the long-term viral reservoir. Intermittently, cells will enter a less restricted form of latency, expressing immunodominant EBNAs leading to their elimination by EBV cytotoxic T cells in the immunocompetent host, or will enter lytic cycle-releasing virus. Recurrent exposure to lytic and latent antigens helps to maintain a strong antigen-specific cytotoxic T-cell response.

As the cellular immune response is established, these proliferating B cells expressing immunodominant viral antigens are targeted for destruction by virus-specific cytotoxic T lymphocytes [6,10]. The lifelong persistence of a subset of infected lymphocytes can be demonstrated by cultivation of peripheral blood mononuclear cells. In the presence of a pharmacologic agent such as cyclosporine that prevents T-cell activation, mononuclear cells give rise to spontaneous EBV-immortalized B-cell lines [11,12]. An estimate of the frequency of infected B cells can be inferred from the number of B cells that must be cultured in order to yield an immortalized B-cell line. In healthy adults, this is usually in the range of 10^4 to 10^6 B cells [13]. Polymerase chain reaction (PCR) yields similar estimates of the frequency of infected cells [6,14]. PCR-based studies also show that in these cells, the viral genome is present as an episome and that viral gene expression is very limited [15–17]. In contrast to the proliferating cells that initially expand the latently infected pool, these cells that persist are not cycling and express very few viral antigens [14,18].

Thus latency is complex, with less restricted and more restricted patterns of viral gene expression that do, and do not, drive the proliferation of infected cells. Neoplastic diseases associated with EBV are inevitably associated with latency. An important consequence is that in contrast to disease associated with lytic herpes virus infections (such as zoster or cytomegalovirus retinitis) that may be controlled by agents that inhibit the viral DNA polymerase, EBV-associated neoplasia is unaffected by these antiviral agents.

Latency has been best studied in EBV-immortalized lymphocytes in vitro. The viral genome is episomal. Six nuclear proteins, three membrane proteins, and two small polymerase III transcripts (the EBERs) are expressed (Figure 2) [2]. Studies done with deleted viral recombinants have shown that immortalization requires the coordinated expression of five viral genes [2]. Among these, EB nuclear antigen 1 (EBNA-1) maintains the viral genome as an episome [19], EBNA-2 activates specific viral and cellular genes, and the latency membrane protein-1 (LMP-1) interacts with tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) that lead to activation of NF-kB and modulation of a variety of apoptotic and growth pathways [20]. In murine cell lines, LMP-1 leads to transformation (loss of contact inhibition, anchorage independence) and tumorigenicity in nude mice [21]. Not required for immortalization but worthy of note is LMP-2A, which inhibits lytic cycle activation by blocking normal B-cell transduction mechanisms, and the EBERs 1 and 2, whose functions are unknown but which have emerged as important markers of EBV latent infection by virtue of their abundance (estimated at 10 million copies per cell) [22–24].

There are several alternative patterns of latency gene expression. Immortalized lymphoblastoid cell lines represent one end of the spectrum. Referred to as *latency III*, the pattern of viral gene expression in immortalized cell lines is also characteristic of B-cell tumors arising in immunocompromised patients, particularly organ and bone marrow transplant recipients [25]. In other tumors, more restricted patterns of viral gene expression have been recognized.

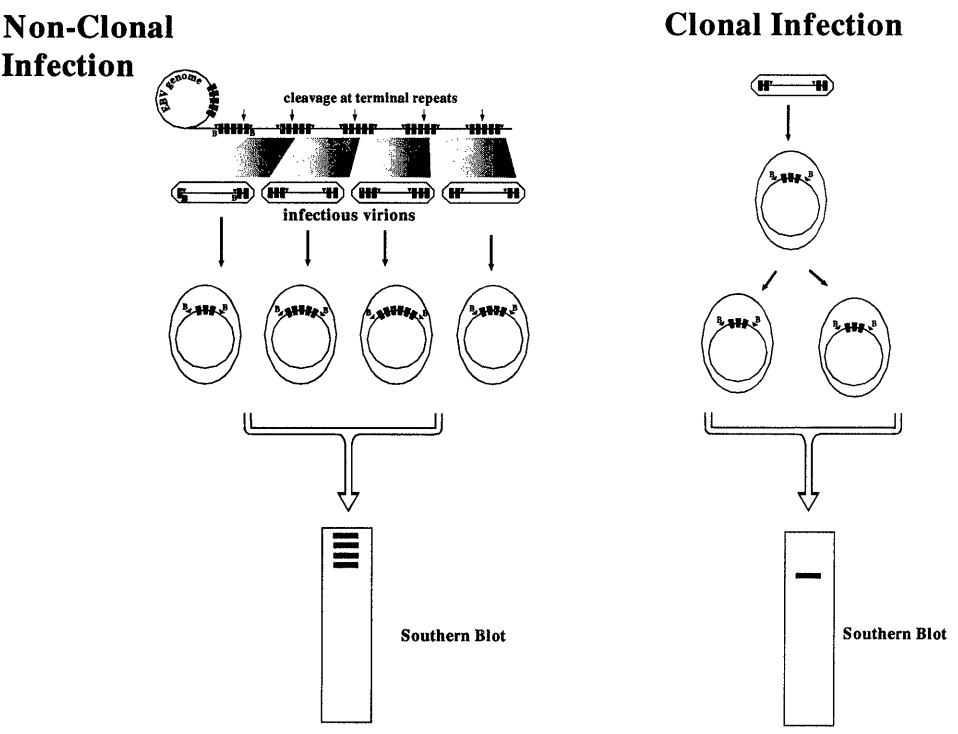

Figure 2. The terminal repeats of the viral genome distinguish polyclonal from monoclonal infected cell expansions. The panel on the left illustrates how the rolling circle mechanism of replication leads to the generation of virion DNA with variable numbers of terminal repeats. These fuse to form episomes. Cleavage of episomal DNA releases terminal fragments consisting of fused right and left ends of the viral genome. Since each infectious event leads to the circularization of a linear viral genome with a particular number of terminal repeats, and since the number of repeats is preserved in latently infected daughter cells, the presence of one or more bands corresponding to the fused terminal repeats distinguishes clonal (one infectious event) from polyclonal infection (multiple infectious events).

In Hodgkin's disease, EBNA-1, LMP-1, LMP-2A, and the EBERs are expressed [26–28]. This pattern is referred to as *latency II.* In Burkitt's lymphoma, only EBNA-1 and the EBERs are expressed, although heterogeneity has been recognized [29]. Termed *latency I*, this same very limited pattern of viral gene expression has also been detected by RT-PCR in peripheral blood lymphocytes from healthy EBV-seropositive individuals.

Lytic infection is detected in only a small minority of lymphoid cells in vitro and in vivo. Lytic cycle is initiated by the expression of ZTA (also referred to as BZLF-1). Expression of delayed early genes, late genes, and ultimately packaging and release of infectious virus follow. Two lytic genes with sequence and functional homology to cellular genes implicated in tumorigenesis are

BHRF-1, a viral BCL-2 homologue, and BCRF-1, a viral IL-10 homologue [30]. These viral proteins share functional properties with their cellular homologues. BHRF-1 inhibits apoptosis, and BCRF-1 alters the character of T-cell responses to antigens. However, any role in tumorigenesis remains conjectural because these viral genes are expressed only in lytic infection, whereas latent infection dominates in tumors [8,31].

Immune response

In traditional immunofluorescence assays, antibody responses to antigen complexes rather than to individual antigens are being measured [32–34]. IgM titers to virus capsid antigen (VCA), and IgG titers to early antigen (EA) appear with primary infection and resolve several months later. IgG titers to VCA appear and persist indefinitely. Titers to nuclear antigens (EBNA) appear late but also persist. Specific assays for particular antigens or peptides have been developed, but these have not yet been broadly applied, particularly in studies of lymphoma patients [35,36].

EBV-specific and nonspecific cellular responses are generated during acute infection. These CD8+ T cells and NK cells are the atypical lymphocytes of infectious mononucleosis. Following primary infection, memory T cells specific for EBV persist at a frequency of approximately 1 in 10^4 circulating T cells. The most frequently targeted antigens are EBNA-3A, -3B, and -3C. EBNA-2, EBNALP, LMP-1, and LMP-2 are also targeted but less frequently [37,38]. EBNA-1 does not serve as a target for CD8+ T cells because a *cis*-acting glycine–alanine repetitive sequence inhibits antigen processing and MHC class I presentation [39].

Characterization and detection

There are certain aspects of EBV biology that provide investigators with distinctive approaches to characterizing these virus-associated tumors. The first relates to inferences regarding the clonality of virus-infected tissues. Whereas in both retroviral and papillomavirus-associated tumorigenesis, viral integration serves to mark clonal expansions of tumor cells, integration of the EBV genome does not occur in lymphocyte immortalization, and integrated viral genomes are not commonly detected in tumors [40]. Nonetheless, the state of the EBV genome has allowed insights into clonality similar to those provided by study of unique integration sites of retroviruses and papillomaviruses [41,42]. In virions, EBV genomes are linear (Figure 2). These linear forms are the products of cleavage of multigenome-length concatemers. Cleavage within the terminal repeat sequences occurs semirandomly such that linear genomes generated from the same parental template differ in numbers of terminal repeats. Upon infection, tandem direct repeats at either end of the linear genomes fuse to form circular episomes. These episomes differ from one another in terms of the numbers of terminal repeats in the fusion joint. In

latent infection, the episomes are replicated as episomes rather than through concatameric intermediates, and numbers of terminal repeats are perpetuated generation to generation. If another cycle of lytic infection ensues, then variation in numbers of terminal repeats occurs again. Thus, when cells that harbor episomes in a tissue are all the clonal progeny of a single latently infected cell, the fusion joint will have a fixed number of terminal repeats. However, when cells that harbor episomes are the progeny of many cells that were infected by different virions (albeit from the same viral source), fusion joints will vary in terms of the number of terminal repeats. These different fusion joints may be recognized by Southern blot hybridization.

In situ localization of virus is particularly important for EBV because the virus is ubiquitous. Detection by PCR and similar sensitive techniques does not readily allow the investigator to differentiate between rare virus infected infiltrating lymphocytes that may contaminate a specimen and the virus specifically associated with tumor cells. Immunohistochemical techniques allow detection of viral antigens, but the highly restricted patterns of viral antigen expression in many tumors limits the general utility of this technique. In situ hybridization has emerged as a standard detection technique because of the abundance of the EBERs (Figure 3) [43,44].

Non-Hodgkin's lymphoma

Posttransplant lymphoproliferative disease and lymphoproliferative disease in congenital immunodeficiency

B-cell lymphoproliferative disorders occur with increased frequency in solid organ and bone marrow transplant recipients as well as in patients with congenital immune deficiency. Virtually all of these are EBV associated [45–47]. There is a histologic spectrum of disease from benign hyperplasia to monoclonal polymorphic proliferations to monomorphic histologies. Hyperplasias are usually polyclonal or oligoclonal, while polymorphic proliferations and monomorphic histologies are usually monoclonal. Polymorphic proliferations and monomorphic histologies typically differ in that the latter are associated with mutations, deletions, or rearrangements of cellular genes [48]. The incidence of these disorders varies with the organ transplanted. Heart–lung transplant recipients and liver transplant recipients have much higher incidences than renal transplant recipients [49]. The details of immunosuppression or of the immunodeficiency syndrome are also important [47,50–52]. The advent of cyclosporine was associated with an increase in the incidence of lymphoproliferative disease, while the ability to monitor cyclosporine levels and thus lower average cyclosporine dosage was associated with a fall in the incidence. OKT3 and other T-cell antibodies have been associated with a particularly high occurrence, whether used to treat organ rejection in solid organ transplant recipients, to treat graft-versus-host disease in bone marrow

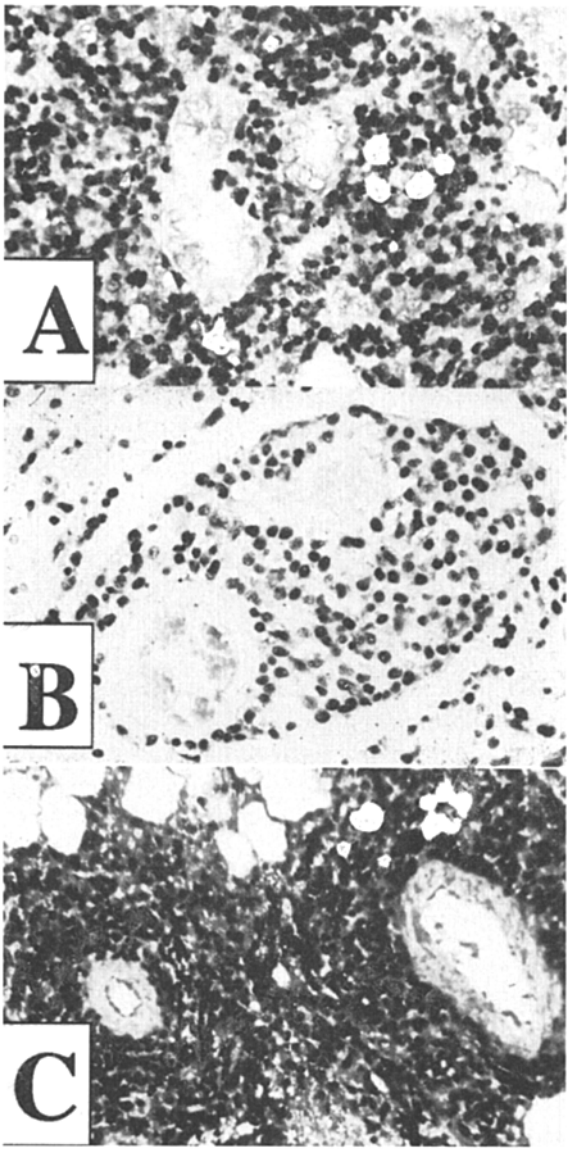

Figure 3. EBER in situ hybridization demonstrates the presence of EBV latency transcripts in posttransplant lymphoproliferative disease arising in the transplanted kidney (**A**), a primary central nervous system lymphoma in an AIDS patient (**B**), and an angiocentric peripheral T-cell lymphoma (**C**). Hybridization with a digoxigenin-labeled antisense riboprobe is detected with an alkaline phosphatase conjugate. EBER signal is nuclear in fixed tissues.

transplant recipients, or to prevent graft-versus-host disease by T-cell depletion of the marrow graft [50,51]. In solid organ transplant recipients, the patient's status with regard to EBV infection at the time of transplant is an important factor. Patients without serologic evidence of prior EBV infection are at greatest risk. Younger age is also a risk factor, although this may simply be a surrogate for lack of prior exposure to EBV. Finally, in the marrow transplant setting, HLA differences between donor and recipient are risk factors.

Posttransplant lymphoproliferative disease is commonly extranodal, frequently arises in the donor organ in solid organ transplant recipients, and usually shows a pattern of latency antigen similar to that of EBV-immortalized lymphoblastoid cell lines (latency III), although there is considerable intratumor and intertumor heterogeneity, with more restricted patterns of antigen expression common [25]. When these tumors arise in solid organ transplant recipients, they are virtually always of host origin. When these tumors arise in allogeneic bone marrow transplant recipients, they are virtually always of donor origin, although exceptions to both generalizations have been reported [53–58]. It is generally accepted that at least some of the tumors that arise in the posttransplant setting simply represent the in vivo outgrowth of EBV-immortalized lymphoblastoid cell lines as a consequence of failed immunosurveillance [31,59].

Treatment approaches that have been advocated include reduction or withdrawal of immunosuppressive therapies, antiviral agents, interferon alpha, anti-B-cell monoclonal antibodies, and conventional chemotherapy. Withdrawal or reduction of immunosuppressive therapy seems to be most effective in lymphoproliferative lesions that develop in the first year after solid organ transplantation [60,61]. Acyclovir or ganciclovir are commonly used [62,63], but lymphoproliferative disease occurs even in patients being treated with these drugs [64,65]. Furthermore, these drugs do not selectively inhibit the proliferation of lymphocytes latently infected by EBV either in vitro or in vivo [66,67]. Anti-B-cell antibodies with specificity for CD21 and CD24 have been ineffective in patients with monoclonal posttransplant lymphoproliferative disease but effective in patients with polyclonal posttransplant lymphoproliferative disease [68]. The possibility that humanized anti-CD20 antibodies might be active in the treatment of posttransplant lymphoproliferative disease is presently being explored.

Dramatic successes have been reported with cellular therapies in the past several years. Patients with lymphoproliferative disease following T-cell-depleted allogeneic bone marrow transplantation have been treated with donor lymphocyte infusion [69]. Regression of monoclonal lymphoproliferative lesions with histologic features of monomorphic, diffuse large cell lymphomas has been reported. Responses were achieved rapidly (14 to 30 days after infusion). In contrast to donor lymphocyte infusion for the treatment of relapsed chronic myeloid leukemia, control of EBV lymphoproliferative disease cannot be attributed to alloreactivity, since lymphoproliferative disease in this

setting regularly occurs in donor rather than host lymphocytes. The high frequency of EBV-specific cytotoxic T-cell precursors in healthy donors is consistent with the interpretation that EBV-reactive T cells play a critical role. Demonstration of the efficacy of donor lymphocyte infusion in the treatment of posttransplant lymphoproliferative disorder in bone marrow transplant recipients was followed by the demonstration that infusions of EBV-specific cytotoxic T cells can also induce regression or prevent the development of tumors [65]. In some cases, populations of cytotoxic T-cell precursors that could respond to in vivo or ex vivo EBV challenge persisted for as long as 18 months [70]. Donor cytotoxic T-cell infusions were not associated with graft-versus-host disease. Used prophylactically in high-risk patients, these infusions appear to be successful in preventing the development of posttransplant lymphoproliferative disease.

Accumulation of genetic alterations such as p53 mutations, rearrangements of c-myc, mutations of n-ras, or alterations in bcl-6 predict a failure to respond to changes in immunosuppressive therapies [46]. These mutations may directly confer resistance to cytotoxic T-cell killing in some instances. Alternatively, the immunodominant EBV genes that drive tumor cell proliferation may be rendered superfluous by mutations in cellular genes. Tumor clones that have downregulated expression of these viral genes may be selected for by residual immune function [59,71].

Burkitt's lymphoma

In contrast to posttransplant lymphoproliferative disorder, where in many instances viral genes clearly drive proliferation and lymphocyte immortalization is a good model for pathogenesis, the association of EBV with Burkitt's lymphoma remains perplexing. The incidence of the tumor is highest in areas of Africa with holoendemic malaria. In these areas the tumor is uniformly associated with the virus, whereas in other parts of the world, the incidence of the tumor and its association with EBV are variable [72,72–74]. Chromosomal translocations that juxtapose the c-myc gene with an immunoglobulin locus, usually the heavy chain locus on chromosome 14, are a constant feature of this lymphoma with or without EBV [73]. However, subtle differences in the distribution of chromosomal breakpoints have been recognized between endemic and nonendemic tumors. What role EBV may play in the generation of these translocations or in the maintenance of cells in which the translocation has occurred is not clear.

What contribution does EBV make to the maintenance of the transformed phenotype in Burkitt's lymphoma? The only viral gene consistently expressed in Burkitt's cells is EBNA-1 [29,75]. Although required for lymphocyte immortalization, this gene has not been shown to affect cell proliferation or resistance to apoptosis. However, two sets of experiments suggest that either EBNA-1 or some other viral function may be important. A contribution of viral episomes to the maintenance of the transformed phenotype has been

demonstrated in a Burkitt's lymphoma cell line [76]. There is ongoing episomal loss from the Akata cell line. Since cells retaining episomes have a growth advantage over those that do not, cells without viral episomes are demonstrable only when individual tumor cells are cloned. Clones of cells that have lost viral episomes grow more slowly than clones retaining episomes and cease to be tumorigenic in murine model systems. A suggestion that EBNA-1 expression might be the relevant viral function comes from a transgenic model in which an EBNA-1 driven by a heavy chain enhancer is associated with the development of B-cell lymphomas [77]. Alternative episomal functions that might be relevant to maintenance of the transformed phenotype include expression of the EBER genes. A role for EBV in sporadic Burkitt's lymphomas has also been entertained following the observation that integrated fragments of viral genomes have been detected in some cases [78].

By contrast with posttransplant lymphoproliferative disease, Burkitt's lymphoma is a tumor that is resistant to immune killing at several levels. The only EBV latent protein expressed is EBNA-1, which is not recognized by cytotoxic T lymphocytes due to an inhibitory effect of repetitive amino acid sequences within the protein on antigen processing [39]. Even if other antigens were expressed, however, the tumor would remain invisible to cytotoxic T cells insofar as essential elements of the class I pathway, including the peptide transporters TAP-1 and TAP-2, are downregulated [79,80]. In some instances, even the class I molecule is not expressed [81]. These observations raise questions as to what role, if any, immunocompromise plays in the pathogenesis of Burkitt's lymphoma. It is worth noting in this regard that although the incidence of Burkitt's lymphoma is dramatically increased in HIV-infected patients, most of these tumors are EBV− [82].

Hodgkin's disease

Epidemiologic evidence has been marshaled to suggest that Hodgkin's disease in young adults might be a rare consequence of a common infection [83,84]. Risk factors for the development of young adult Hodgkin's disease include small family size, individual housing, and maternal education — all of which are associated with delayed exposure to common viral infections. Cohort studies of patients with serologically documented infectious mononucleosis consistently show a two- to fourfold excess of Hodgkin's disease [85,86]. Furthermore, serologic studies show higher mean antibody titers to viral antigens in patients with Hodgkin's disease than in controls. The serologic differences are most marked several years before the diagnosis of Hodgkin's disease [87].

Viral DNA is detected in Hodgkin's disease by blot hybridization studies in 20%–30% of cases [88–91]. Analysis of the terminal repeats shows a single band, indicating that the virus is present prior to clonal expansion [89–91]. Originally, the possibility was entertained that viral genomes might be present in one of the infiltrating benign cell populations of the polymorphous infiltrate.

However, in situ hybridization and antigen detection studies localized the virus to Reed–Sternberg cells and their variants [90–94]. Under optimized conditions, both detection systems yield nearly identical results [95–98]. EBNA-1 and LMP-2A have also been demonstrated in EBV-positive cases [27]. The other EBNAs are not expressed. Thus, Hodgkin's disease demonstrates a latency II expression pattern. Histologic type, age, sex, and ethnicity all affect the association of EBV with Hodgkin's disease. Glaser et al. compiled data on 1546 Hodgkin's disease patients from 14 studies [99]. The mixed cellularity subtype (vs. nodular sclerosis), young adult male (vs. female), Hispanic (vs. white), and children from economically less developed versus more developed regions were all associated with an increased risk of EBV+ Hodgkin's disease. Whereas the percentage of Hodgkin's disease cases in North America and Western Europe that are EBV associated is less than 50% in virtually all series, the percentage in some populations, such as children in Honduras, Peru, and Kenya, is greater than 90% [100–102]. In HIV-infected patients, virtually all cases are EBV associated. As noted below, this rate of EBV association is higher than the rate in non-Hodgkin's lymphomas in this same population [103–105]. Familial Hodgkin's disease is less likely to be EBV associated than sporadic Hodgkin's disease [106,107]. Curiously, no correlation has been demonstrated between the presence or absence of EBV in Hodgkin's tumor cells and patient serology [34,108].

With regard to potential susceptibility to immune manipulations, it has been observed that Reed–Sternberg cells are heterogeneous in their expression of MHC class I antigens [109]. Curiously, EBV− cases of Hodgkin's disease but not EBV+ cases show MHC class I downregulation [110]. Furthermore, TAP-1 and TAP-2 are consistently expressed. Studies with cell lines putatively derived from Reed–Sternberg cells demonstrate sensitivity to killing by antigen-specific cytotoxic T cells [111]. Thus, while antigen expression in Hodgkin's disease is restricted to EBNA1, which is not targeted by subdominant cytotoxic T cell responses, and to LMP-1 and -2, which are targeted, there is no evidence that tumor cells in EBV+ Hodgkin's disease are inherently resistant to class-I-mediated killing. However, it has been suggested that local production of cytokines and chemokines may inhibit cytotoxic effector cell function [112,113].

HIV-associated lymphomas

Among HIV-infected individuals, there is an increase in the incidence of lymphoma. Approximately half of these cases are EBV+ [114–116]. The association is nearly universal in primary central nervous system lymphomas (Figure 3B) and in other tumors that tend to occur in individuals with long-standing AIDS who have extremely low T-cell counts.[117] Primary central nervous system lymphoma is often a diagnostic challenge. However, several studies have shown that EBV can be fairly consistently detected in the cerebrospinal fluid of these patients and only rarely in HIV patients without

primary central nervous system lymphoma [118–120]. In combination with radiologic studies, EBV PCR may make brain biopsy unnecessary in many cases. Other AIDS lymphomas, particularly Burkitt's, or large cell lymphomas occurring in nodal sites in individuals with earlier-stage AIDS, are only occasionally EBV associated [116]. Primary effusion lymphomas, in which the tumor cells are found in serosal cavities without the formation of tumor masses, consistently show dual infection with EBV and HHV-8, the Kaposi's sarcoma-associated herpes virus [121,122]. As noted above, the incidence of Hodgkin's disease is increased in HIV-infected patients, and in this setting the tumor is virtually always EBV associated [103–105].

Other lymphomas

Peripheral T-cell and NK-cell lymphomas are also sometimes associated with EBV (Figure 3C). Nasal lymphoma, previously referred to as lethal midline granuloma, is common in Asian populations but rare in Western populations. CD56+ tumors, indicating an NK-cell derivation, are always EBV associated, while CD56− tumors are more variable in their association with EBV [123–128]. These tumors consistently express LMP-1 and are clonal by analysis of terminal repeat sequences. Lymphomatoid granulomatosis histologically resembles nasal lymphoma but is a B-cell malignancy with heavy T-cell infiltration. These tumors are also commonly EBV+. Angioimmunoblastic-like peripheral T-cell lymphoma and most other peripheral T-cell lymphomas are sometimes EBV+, although the virus is generally detected in only a subset of the neoplastic population [129,130].

Conclusion

EBV, first discovered in Burkitt's lymphoma, has been found to be associated with a variety of other lymphomas. As these new associations have been recognized, as many questions as answers have resulted. Except in the posttransplant setting, the pathogenesis of these tumors and the explanation for identified risk factors seem elusive. It seems likely that the era of recognition of new EBV associations is drawing to a close. Experience with posttransplant lymphoma illustrates the promise of novel approaches to tumor therapy that specifically target the virus in the future.

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3. Therapy of diffuse aggressive lymphomas

Yener Koc and David P. Schenkein

The non-Hodgkin's lymphomas (NHLs) represent a heterogeneous group of disorders linked together by the commonality of a malignant monoclonal population of either B or T lymphocytes but differing in clinical presentation, prognosis, and treatment strategies. In 1997, the estimated number of new cases of NHL was 53,600, with a slight preponderance toward males (M/F ratio: 1.3) [1]. The estimated number of deaths secondary to NHL was 23,800 (44.4%) in the same year. Non-Hodgkin's lymphoma is among the top 10 leading cancers in females and males, constituting 4% of newly diagnosed cancers in both sexes [1]. Among the reported deaths due to cancer in the United States, NHL is the second most frequent cause in males in the 15–34 age group and the third most frequent cause in the 35–54 age group [1]. The most common aggressive lymphoma is diffuse large B-cell lymphoma, accounting for 60%–70% of cases in the United States and Europe. The focus of this chapter will be the review of the treatment approaches for aggressive lymphomas as defined by both the Working Formulation (WF) and the Revised European-American Lymphoma (REAL) classification systems [2]. Treatment for the follicular lymphomas will be covered elsewhere.

Biology, classification, and prognostic factors

The Working Formulation classification splits the lymphomas into three groupings, with intermediate- and high-grade histologies considered aggressive (Table 1). At present, several variants of NHL are not included in the Working Formulation, e.g., anaplastic large cell type, T-cell rich B-cell type, peripheral T-cell type. The REAL classification, by linking together similar immunologic and clinical characteristics of these lymphomas, has moved the intermediate- and high-grade histologies together into an aggressive category (Table 2). Aggressive lymphomas include diffuse large B-cell lymphomas (LCLs), anaplastic large cell lymphoma (ALCL), and a variety of peripheral T-cell lymphomas (PTCLs). Highly aggressive lymphomas are defined as a clinical subgroup of tumors that are likely to be fatal in weeks if left untreated. These consist of precursor B- and T-lymphoblastic leukemia/lymphomas (B-

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Table 1. Working Formulation classification for the non-Hodgkin's lymphomas [102]

Low grade	Intermediate grade	High grade
Small lymphocytic	Follicular large cell	Immunoblastic large cell
Follicular small cleaved cell	Diffuse small cleaved cell	Lymphoblastic
Follicular mixed cell	Diffuse mixed cell	Small noncleaved cell (Burkitt's or non-Burkitt's)
	Diffuse large cell	

Table 2. A summary of the Revised European–American Lymphoma (REAL) classification proposed by the International Lymphoma Study Group [2]

B-cell lymphoma	T-cell lymphoma	Others
B-cell lymphoblastic	Precursor T lymphoblastic	Composite lymphoma
Small lymphocytic	Adult T-cell lymphoma/leukemia	Lymphoma, unclassifiable, low grade
Lymphoplasmacytoid	Large granular lymphocyte	Lymphoma, unclassifiable, high grade
Mantle cell	Mycosis fungoides/Sezary	Hodgkin's disease
Follicular center, grade 1	Angioimmunoblastic	Anaplastic large cell Hodgkin's-like
Follicular center, grade 2	Peripheral T medium sized	
Follicular center, grade 3	Peripheral T large cell	
Follicular center, diffuse, small	Peripheral T mixed	
Marginal zone, MALT	Peripheral T hepatosplenic	
Marginal zone, nodal	Peripheral T panniculitic	
Marginal zone, splenic	Peripheral T lymphoepithelioid	
Hairy cell leukemia	Angiocentric	
Plasmacytoma	Lymphomatoid granulomatosis	
Diffuse large cell	T-cell CLL/prolymphocytic	
Burkitt's	Anaplastic large cell	
Burkitt's-like		

LBL and T-LBL), adult T-cell lymphoma/leukemia associated with HTLV-I infection, and Burkitt's lymphoma [3].

In an attempt to understand the biology of the aggressive non-Hodgkin's lymphomas, several recently defined biological markers have been demonstrated to correlate with prognosis and clinical outcome. Of these, markers measuring the proliferative activity of the malignant cells, suppression of apoptosis, and cytokines may be the most useful. These include measurement of S-phase fraction by DNA flow cytometry, determination of Ki-67, and bcl-2 expression. Overexpression of these factors is associated with a poor prognosis [4–6]. Other potential biological markers include soluble TNF, CD44, β-2 microglobulin, interleukin-6, and interleukin-10 [7–12]. The recently defined International Prognostic Index (IPI) utilizes clinical criteria and is valuable in designing and comparing different treatment regimens for the aggressive lymphomas (see below and Tables 3a and 3b) [13].

Table 3a. Clinical criteria for assessment of the International Prognostic Index [13]

All patients	Age adjusted (<60)
Stage III, IV	Stage III, IV
Performance status 2,3,4	Performance status 2,3,4
LDH > normal	LDH > normal
Age >60	
# Extranodal sites >1	

Table 3b. Complete remission and survival rated in patient groups classified according to the International Prognostic Index (IPI) [13]

Risk group	No. of IPI risk factors	% CR	RFS (5-yr) all pts	OS (5-yr) all pts	OS[a] (5-yr) (age < 60)	OS[a] (5-yr) (age > 60)
Low	0–1	87	70	73	83	56
Low-intermediate	2	67	50	51	69	44
High-intermediate	3	55	49	43	46	37
High	4–5	44	40	26	32	21

[a] Age-adjusted model.
Abbreviations: RFS, relapse-free survival; OS, overall survival.

Determination of patients with high-risk disease

The most commonly used model for determining risk profile at the time of diagnosis is the international index, which is based on five independent variables — age, LDH level, performance status, number of sites with extranodal involvement, and stage of the disease (Tables 3a and 3b) [13]. The age-adjusted index, designed for patients under the age of 60, utilizes stage, LDH, and performance status as the prognostic variables [13]. Only 26% of patients with high-risk disease have a chance for long-term survival when treated with the standard induction regimens. Overall survival is negatively impacted by a decreased complete remission rate and an increased relapse rate in the high-risk groups. This index serves as a guide for risk-based therapeutic decisions and a guide for clinical trials design (see Table 3b).

Clinical criteria other than the parameters used in the international prognostic index have also been evaluated. These include treatment-related prognostic factors such as time to achieving a complete remission [14], the dose of doxorubicin and cyclophosphamide received in the first 12 weeks of therapy [15], and cellular/biomolecular markers reflecting the biology and aggressiveness of the disease. Evaluation of response to the induction regimen may be assessed more precisely by Gallium-67 citrate imaging, since computerized tomography (CT) scans often detect residual nontumor-associated masses in

patients presenting with bulky disease. Persistence of Gallium positivity after the patient receives four cycles of the induction regimen significantly reduces the chance of having a durable response (24% vs. 70%) [16]. If a negative Gallium scan is obtained after two cycles of a dose-intensive regimen, the likelihood of inducing a durable remission is higher [17].

Additional molecular and cellular markers such as high levels of Ki-67 nuclear proliferation antigen [18], T-cell phenotype [19], lack of HLA-DR antigen expression [20], decreased number of $CD8^+$ tumor-infiltrating lymphocytes [21], increased β_2-microglobulin levels [22], CD44 adhesion molecule expression [23], karyotypic abnormalities involving the short arm of chromosomes 17 and 7 [24], and overexpression of bcl-2 [25] have been reported to be associated with poorer overall survival and increased recurrence rates. Individuals carrying these molecular or phenotypic abnormalities may be considered to have high-risk disease, although they are not included in the international criteria. Of these, the Ki-67 antigen, measured by a monoclonal antibody applied to frozen sections, closely correlates with median survival and is an independent prognostic factor in multivariate analysis [18]. Patients who have greater than 60% Ki-67+ cells have a median survival of eight months, compared to 39 months in the patient group showing lower expression of this antigen [18].

Historical perspective to treatment

High-grade NHL was initially shown to be a malignancy curable with the combination chemotherapy of C-MOPP (cyclophosphamide, vincristine, procarbazine, and prednisone), soon replaced by the CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen in the 1970s [26]. Although there had been an apparent improvement in survival reported with 'third'-generation treatment regimens [1,27,28], these differences in comparison to CHOP were not validated in randomized trials. The overall cure rates reported with the CHOP regimen in diffuse large cell lymphoma generally range between 35% and 45%. Single-institution studies aiming to improve cure rates by using multiagent third-generation regimens showed 70% to 85% complete response and 60% to 70% survival rates; however, additional complete responders did not become long-term disease-free survivors, and the survival advantage disappeared during long-term follow-up [27]. The Intergroup trial compared standard therapy (CHOP) to the third-generation chemotherapy regimens (m-BACOD, ProMACE-CytaBOM, or MACOP-B) in the diffuse aggressive lymphomas. No difference in response rate or overall survival between CHOP and the third-generation regimens was noted [28]. CHOP remains the standard of care, given its ease of administration, reduced cost, and lower toxicity profile.

Historically, patients with early-stage disease (aggressive histology) were treated with radiotherapy alone. The efficacy of this strategy was heavily

dependent on the accuracy of the staging techniques [29]. As systemic chemotherapy was introduced into management of stage I and II disease, the use of staging laparotomy and large-field radiation therapy declined [30,31]. Recent data reported by the Eastern Cooperative Oncology Group (ECOG) suggest that adjuvant involved field radiotherapy is beneficial following eight cycles of CHOP in the management of bulky or extranodal early-stage disease [32]. In this randomized phase III study, patients receiving 3000 cGy of adjuvant radiotherapy had a significantly better disease-free survival (DFS; 73% vs. 58%) and overall survival (OS; 84% vs. 70%) compared to patients treated with CHOP alone. Similarly, SWOG has conducted a randomized trial of CHOP for three cycles plus radiation versus CHOP for eight cycles for early-stage disease. This trial demonstrates a survival advantage for patients treated with the combined-modality approach, with lower toxicity [33]. In other nonrandomized studies reported from the National Cancer Institute and Vancouver, 47 of 49 patients with nonbulky stage I or IE aggressive lymphoma remained disease-free at four years after receiving four cycles of PROMACE-MOPP regimen followed by involved field radiotherapy [34] or three cycles of CHOP followed by involved field radiotherapy without compromise in DFS (84% at three years) [35], respectively. Combined-modality treatment should be considered the standard of practice of early-stage aggressive lymphomas.

New approaches to initial treatment of aggressive lymphomas

The ability to achieve a cure is strongly affected by age, tumor burden, and biology of the tumor as reflected by the cell turnover rate, as demonstrated by use of the International Prognostic Index IPI [13]. IPI provides valuable prognostic information based on clinical criteria and is widely used to stratify patients with high-grade non-Hodgkin's lymphoma patients into clinical trials designed to improve the cure rates in certain subgroup of patients with poor prognosis.

Since the standard CHOP regimen cures less than 40% of unselected patients [36], more effective treatment approaches are needed to improve the outcome in certain subgroups of patients with aggressive lymphoma. Current strategies to improve outcome include exploiting dose intensity, high-dose chemotherapy with or without stem-cell support, newer chemotherapy agents, and immunotherapy. Patients who have poor-prognostic features at the time of diagnosis are potential candidates for clinical trials evaluating new treatment modalities.

Role of dose intensity

Dose intensity, defined as the amount of chemotherapy delivered per unit time [37], is an important factor in treatment outcome for the aggressive lymphomas [38]. In the French GELA study, patients receiving less than 70%

of the planned dose intensity had a significantly poorer outcome [39]. Dose intensity was found to be an independent prognostic factor in patients receiving CHOP, m-BACOD, and MACOP-B regimens in a retrospective multivariate analysis of the Stanford series [15], but small increments in doxorubicin dose did not yield a significant benefit when corrected with other prognostic factors, as demonstrated in a Canadian trial [40]. Use of hematopoietic growth factors to increase or maintain the dose intensity has not translated into improved survival, although some benefit in decreasing the frequency of neutropenic fevers and treatment delays have been demonstrated [41,42].

Dose-intensified induction regimens without stem cell support

Significant escalation in doxorubicin and cyclophosphamide doses in the CHOP regimen have been tested to improve the complete remission and survival rates. The Dana Farber Cancer Institute mega-CHOP regimen and Vanderbilt multidrug high-intensity regimen tested this hypothesis [43,44]. In the mega-CHOP dose finding study, patients with bulky disease (mass >10 cm) received a dose-escalated CHOP. At the maximum tolerated dose level, patients received four 21-day cycles of cyclophosphamide (4 g/m^2), doxorubicin (70 mg/m^2), vincristine (2 mg), and prednisone (100 mg) with growth factor support. This nonrandomized prospective trial resulted in a complete remission (CR) rate of over 80%. However, the duration of median follow-up is still too premature to draw any conclusions about the impact of CHOP dose escalation on long-term survival. Hematologic toxicity was significant, with one treatment-related death and a 65% incidence of midcycle febrile neutropenia [43]. In the Vanderbilt multidrug high-intensity program, the major adverse events were related to myelosuppression, with a 100% incidence of neutropenic fevers, 59% severe thrombocytopenia, 27% severe mucositis, and 10% deaths related to myelosuppression [44]. The toxic deaths were primarily in older patients (>60 years of age). The CR rate was 81%, with a five-year actuarial survival of 52%. The Eastern Cooperative Oncology Group recently tested the ProMACE-CytaBOM regimen delivered at twice the standard dose. Treatment for all prognostic groups was well tolerated and effective, with DFS of 60%–80% and OS of more than 80%, adjusted for risk (Gordon, personal communication). None of these dose-intense treatments has been compared to CHOP in a randomized setting.

Consolidation with high-dose chemotherapy and stem cell support

High-dose chemotherapy with either bone marrow or peripheral blood stem cell support has been tested as consolidation of either a partial or a complete remission in selected patient populations (Table 4).

The French Lymphoma Trials Group (GELA) performed a randomized trial designed to compare sequential conventional-dose chemotherapy to au-

Table 4. Results of randomized trials of autologous transplantation as initial therapy

Study design	Timing of BMT	No. of patients	CR (%)	DFS (%)	OS (%)	Ref.
CHOP vs. CHOP and ABMT	1st PR	69	74 vs. 68	72 vs. 60	85 vs. 56	49
DHAP vs. ABMT: after F-MACHOP or MACOP-B	1st PR	49	15 vs. 14	52 vs. 73	59 vs. 73	50
Sequential consolidation vs. ABMT: after ACVB	1st CR	236	n/a	39 vs. 59 ($p = 0.01$)	52 vs. 65 ($p = 0.06$)	163
MACOP-B vs. HDS	Initial therapy	98	70 vs. 96 ($p = 0.001$)	49 vs. 84 ($p = 0.001$)	55 vs. 81 ($p = 0.09$)	51
ACVB vs. induction/ABMT	Initial therapy	370	64 vs. 63	54 vs. 41 ($p = 0.01$)	63 vs. 47 ($p = 0.003$)	46

Abbreviations: CR, complete remission; DFS, disease-free survival; OS, overall survival. For study designs, see text.

tologous bone marrow transplantation (BMT) as consolidation in patients who have achieved CR from standard induction therapy. Analysis of the entire cohort did not reveal a significant difference in either disease-free or overall survival [45]. In a subsequent analysis of 236 poor-risk patients (two or three risk factors), disease-free survival in the autologous BMT arm (59%) was found to be superior to sequential therapy-based consolidation (39%). The difference in overall survival at five years was relatively small, but significant (65% vs. 52%, $p = 0.06$) [47]. This prospective trial supports high-dose consolidation therapy with autologous stem cell support in younger high-risk patients who have achieved a complete response with a standard induction regimen.

In a subsequent GELA study, 370 patients less than 60 years of age with high-risk (IPI 2 and 3) disease were randomized to standard therapy (ACVB and sequential consolidation) and a new intensified induction course (CEOP, ECVBP, and ECVBP) followed by BEAM and PBSC rescue [46]. Although the complete remission rates were similar, the transplant arm had significantly lower overall and event-free survival than the conventional-therapy group.

Vitolo and colleagues performed a nonrandomized pilot trial using early intensification with autologous BMT in patients with high-risk large cell NHL and demonstrated a 50% DFS rate at a median follow-up of 32 months with a 6% toxic death rate [47]. Stoppa and colleagues utilized three cycles of dose-augmented CHOP to facilitate PBSC collection followed by three courses of PBSC-supported cisplatin and etoposide in the outpatient setting for high-risk patients. The two-year overall survival and relapse-free survival were 73% and 56%, respectively [48].

Verdonk and colleagues evaluated the impact of early high-dose consolidative therapy in patients who failed to achieve a complete remission

following three cycles of CHOP therapy. Patients who achieved a partial remission after three cycles of CHOP were randomized to high-dose cyclophosphamide combined with single-dose total body irradiation (TBI) and stem cell rescue or an additional five cycles of the CHOP regimen [49]. The majority of patients enrolled into this trial had low- and low–intermediate-risk disease. The study failed to demonstrate any benefit from consolidative high-dose chemoradiotherapy in first partial remission.

In a smaller, randomized study designed to answer a similar question, partial responders to conventional induction treatment received either a DHAP salvage regimen (27 patients) or a high-dose BEAC regimen followed by an autologous bone marrow rescue (22 patients) [50]. Although the patients randomized to the high-dose chemotherapy arm had a 73% DFS rate compared to 53% observed in patients receiving the DHAP salvage regimen, a statistically significant advantage for transplant was not achieved [50]. Thus, additional clinical trials are needed to define the role of high-dose chemotherapy in patients failing to achieve a complete response from standard induction therapy.

High-dose chemotherapy with stem cell support as initial therapy

Intensifying the initial treatment regimen with stem cell support is a new approach designed to improve outcome in patients who are at high risk for recurrence. In a recent randomized trial, Gianni and colleagues treated 98 high-risk patients with either m-BACOD or high-dose sequential chemotherapy (HDSC) including cyclophosphamide, methotrexate, and etoposide followed by L-PAM/TBI and peripheral blood stem cell transplant (PBSCT) [51]. The majority of patients had diffuse large cell histology and those with marrow involvement were excluded. At 55 months median follow-up, freedom from progression was 84% in the high-dose therapy arm compared to 49% in the m-BACOD arm, and an improved survival in the HDSC/PBSCT arm (81%) over conventional induction chemotherapy (55%) was demonstrated ($p = 0.09$). A recently reported phase II, prospective, multicenter trial of a similar high-dose sequential chemotherapy regimen and peripheral blood stem cell transplantation as initial treatment for patients with high-risk NHL demonstrated a CR rate of 95% with an overall survival of 82% and relapse-free survival of 78% at 18 months follow-up [52]. Sequential therapy consisted of cyclophosphamide, methotrexate, etoposide, mitoxantrone, and melphalan used at maximally tolerated doses. Further follow-up is needed to assess the long-term DFS rate that will be achieved with this regimen. The role of initial treatment using high-dose chemotherapy and stem cell support in high-risk patients remains controversial. An ECOG phase III randomized trial of high-dose sequential chemotherapy versus CHOP chemotherapy in intermediate-grade high-risk NHL is currently under way.

New approaches to treating relapsed aggressive lymphomas

Despite initial treatment, a significant number of patients with aggressive lymphoma are destined to relapse after induction therapy. A variety of strategies used at the time of recurrence have been designed and tested. Despite these treatments, the ability to cure relapsed patients remains a major challenge.

Conventional-dose salvage chemotherapy

Treatment protocols known as DHAP, CEPP, ESHAP, IMVP-16, and IIVP-16 generally incorporate etoposide, cytarabine, cisplatin, ifosfamide, methotrexate, and idarubicin — agents not typically used in the first-line programs [53–58] (see Table 5). With these salvage regimens, an average of 25% to 35% % of patients achieve a second CR, which is generally not durable. There are few controlled trials directly comparing different second-line therapies and little evidence that a major difference exists between them. Prolonged infusional chemotherapy has been tested in an attempt to overcome drug resistance. In a study employing a 96-hour infusion of doxorubicin, vincristine, and etoposide in combination with bolus cyclophosphamide and oral prednisone (EPOCH), a CR rate of 27% and a partial remission (PR) rate of 60% was obtained in patients with relapsed or refractory disease [59]. Unfortunately, the response was not durable, with a median survival of between 6 and 21 months depending on the number of prior chemotherapy agents utilized. Many responding patients ultimately proceeded to transplantation. An event-free survival rate of 28% at one year was reported in this study. In a similar approach, Sparano and colleagues tested the efficacy of infusional cyclophosphamide, doxorubicin, and etoposide (CDE) in patients with relapsed or resistant intermediate- or high-grade non-Hodgkin's lymphoma. An objective response was observed in 52% of patients, with a 17% CR rate [60]. Similar to the EPOCH regimen, the duration of response was brief, as re-

Table 5. Results for conventional-dose salvage chemotherapy

Regimen	No. of patients	CR (%)	PR (%)	Duration of response (months)	PFS (%)	Ref.
DHAP	39	23	44	7.5	15% @ 3 yr	53,54
ESHAP	122	37	27	20	28% @ 3 yr	55
CEPP	42	31	36	7	4% @ 3 yr	56
IMVP-16	52	37	25	12	17% @ 3 yr	57
IIVP-16	38	21	26	7	37% @ 2 yr	58
EPOCH	53	34	49	13	0% @ 3 yr	59
CDE	47	21	47	4	10% @ 2 yr	60

Abbreviations: CR, complete remission; PR, partial remission; PFS, progression-free survival. For regimens, see text.

flected by a 19% progression-free and 10% disease-free survival with a median follow-up of two years [60]. Despite introduction of different chemotherapy agents and an altered administration schedule, newer conventional-dose salvage regimens are plagued with high recurrence rates and have failed to induce long-term survival in a vast majority of patients with recurrent disease.

High-dose therapy and autologous transplantation for relapsed disease

High-dose salvage chemotherapy coupled with marrow or stem cell rescue has been extensively utilized in the management of relapsed/refractory patients with aggressive lymphoma following conventional-dose salvage chemotherapy. Several retrospective trials suggested a survival advantage for patients receiving high-dose chemotherapy and demonstrated a prolonged survival compared to conventional salvage chemotherapy [61–68]. With this approach, durable responses or cure may be achieved in certain subsets of patients.

The recently completed, prospective randomized PARMA trial [69] tested the efficacy of autologous bone marrow transplantation versus salvage chemotherapy in chmosensitive relapsed patients. In this study, 215 patients in first or second relapse received two cycles of the DHAP regimen. Subsequently, 109 patients who had a response to chemotherapy were randomly assigned to receive four courses of DHAP plus radiotherapy (54 patients) or radiotherapy plus intensive chemotherapy and autologous bone marrow transplantation (55 patients). The two groups were well balanced for their prognostic factors. Complete response rates in the high-dose therapy/BMT and conventional chemotherapy groups were 84% and 44%, respectively. Event-free survival at five years was 46% in the BMT group and 12% in the group receiving chemotherapy alone ($p = 0.001$), with overall survival at 53% and 32%, respectively ($p = 0.038$) [69]. These results demonstrate the efficacy of autologous transplantation in chemosensitive relapsed patients and establish this modality as the standard of care.

Relapse will ultimately still occur in less than 50% of patients receiving high-dose therapy. It remains unclear if this relapse rate is secondary to occult tumor in the graft or from residual bulk disease in the host. A high rate of occult lymphoma involvement in both bone marrow and peripheral stem cell collections has been demonstrated [70]. However, the effect of tumor cell contamination of the graft on long-term survival or cure rates remains unknown.

Treatment of elderly patients with aggressive lymphoma

In the context of the management of aggressive lymphomas, the term *elderly* typically defines the group of patients greater than 60 years of age. Age does

not appear to alter the CR rate; however, elderly patients have an increased relapse and disease-related mortality rate, leading to a significant deterioration in overall survival rates within each prognostic group (Table 3b) [71–73]. The deleterious effect of advanced age on overall survival is less prominent in the intermediate and high-risk patient groups. Despite higher toxicity related to age, the reanalysis of the intergroup trial (SWOG-8516, INT 0067) supports the use of full-dose chemotherapy in the high-risk subgroup of elderly patients [74–76]. The EORTC has recently reported on a randomized trial of CHOP versus VMP (etoposide, mitoxantrone, and prednimustine) and demonstrated an advantage for CHOP in both progression-free and overall survival at two years follow-up [77]. While the optimal treatment of aggressive lymphoma in the elderly is a subject of ongoing debate, patients with high-intermediate and high-risk disease should be treated with full-dose conventional induction regimens [78]. Substitution of doxorubicin with mitoxantrone in the CHOP regimen in an effort to improve tolerance to chemotherapy and reduce the risk of cardiotoxicity has resulted in lower CR and overall survival rates and is not generally recommended [79].

Modification of the CHOP regimen schedule, in an attempt to improve tolerability, failed to increase dose intensity and was associated with a decreased overall survival rate [75]. Other abbreviated regimens have resulted in an average overall survival of 35%–40% at 4 or 5 years, which is comparable to the rates obtained with conventional induction regimens [80–85]. These modified regimens may have a role in elderly patients who cannot tolerate or receive the CHOP regimen.

Treatment of specific subgroups of aggressive lymphomas

Burkitt's and Burkitt's-like lymphomas (SNCL)

Small noncleaved cell lymphoma (SNCL) is a high-grade, biologically aggressive neoplasm notable for poor response to therapy, high relapse rate, and fewer than 20% long-term survivors. Burkitt's and Burkitt's-like lymphomas compose less than 1% of the adult lymphomas [86]. Histologic distinction between Burkitt's and Burkitt's-like lymphomas is based on the degree of cellular pleomorphism and the proportion of cells with a single large nucleolus. The distinction between these two groups are the uniformity (Burkitt's) or heterogeneity (Burkitt's-like) of the nuclear size and contour [87] and the absence of c-myc rearrangements in Burkitt's-like histology [88]. Patients with Burkitt's lymphoma are younger (median age 31 vs. 56 years) and more frequently present with extranodal involvement [87]. Chromosomal translocations are common, with 72% carrying the t(8;14), 20% the t(8;22), and 8% the t(8;2) [89,90]. The common feature of these aberrations is translocation of the c-myc oncogene located on chromosome 8 with the immunoglobulin genes

present on chromosome 14 (heavy chain), 22 (l light chain), and 2 (k light chain) [89,90].

Adults with SNCL are typically treated with chemotherapy protocols based on the regimens used in the management of children with this disease, which include high-dose combination chemotherapy followed by CNS prophylaxis [91–94]. A high-intensity, short-duration regimen consisting of doxorubicin, vincristine, etoposide, bleomycin, methotrexate, and prednisone demonstrated a CR rate of 85% and a DFS of 65% at 2.5 years in adults with advanced-stage SNCL [95]. A retrospective analysis has demonstrated that French pediatric protocols were tolerable and highly effective in 65 adults with SNCL [96]. These protocols consist of an initial phase of cyclophosphamide, vincristine, and prednisone for cytoreduction, followed by an induction regimen including cyclophosphamide, doxorubicin, vincristine, high-dose methotrexate, and prednisone. Consolidation chemotherapy consists of either high-dose methotrexate and cytarabine or etoposide and combined high-dose systemic and intrathecal cytarabine.

Allogeneic BMT in adults with high-risk Burkitt's lymphoma in first CR has been attempted, with promising results. Seven of nine patients remain alive and disease-free at 18–59 months [97]. High-dose chemotherapy coupled with autologous marrow/peripheral stem cell transplantation in patients with high-risk disease as defined by marrow and/or CNS involvement at diagnosis remains controversial at present.

Lymphoblastic lymphoma (LBL)

The term *lymphoblastic lymphoma* (LBL) was first used in 1976 to define a separate clinical entity [98]. LBL cells are immature lymphoid cells with fine chromatin and indistinct nucleoli that are indistinguishable from the cells of acute lymphoblastic leukemia (ALL). They can be divided into two subgroups: those with and those without convoluted nuclei [98]. Unlike most aggressive lymphomas, 80% to 90% of adult LBLs typically display a T-cell phenotype with an intermediate degree of thymic differentiation [99,100]. There is considerable clinical overlap between LBL and T-cell ALL, with mediastinal and nodal features more common in LBL instead of the blood and marrow features commonly seen in T-cell ALL [101]. LBL is primarily a disease of children and young adults, with a second peak incidence above age 40. LBL accounts for 4% of patients with aggressive lymphoma [102]. A mediastinal mass is a presenting sign in 75% of the patients [103]. Although marrow involvement is frequent in LBL, marrow dysfunction leading to pancytopenia or very high white cell counts with circulating blasts are uncommon. There is an increased risk of CNS involvement; however, recurrent CNS disease is rare (3%) if appropriate CNS prophylaxis is administered during or soon after the induction therapy [101,103]. While initial series of adult LBL indicated a very poor prognosis, with a median survival of 6–9 months and few

long-term survivors [101], recent therapeutic advances have made long-term survival possible in approximately 50% of adults [103–107].

The modified LSA2–L2 regimen consists of cyclophosphamide, vincristine, prednisone, and adriamycin induction with mediastinal radiotherapy and intrathecal methotrexate followed by cytarabine, 6-thioguanine, L-asparaginase, and CCNU consolidation. Cranial irradiation and further intrathecal methotrexate is also utilized. In this regimen, maintenance treatment consists of cyclical chemotherapy and intrathecal methotrexate, continuing for a total of three years. This regimen resulted in a 73% CR rate, with an actuarial survival rate of 40% at five years [105].

Additional clinical trials have demonstrated the relatively favorable outcome observed in patients treated with cyclophosphamide, doxorubicin, vincristine, prednisone, and methotrexate induction, followed by CNS prophylaxis, and consolidation therapy followed by maintenance with oral methotrexate and 6-mercaptopurine for a total of 12 months of treatment, similar to the regimens used in the treatment of ALL [103]. Following treatment, patients with favorable prognostic factors had a five-year disease-free survival rate of 94% compared to 19% in those with adverse factors. Other combinations, including a CHOP-based regimen (CHOP/L-asparaginase) with CNS prophylaxis (intrathecal methotrexate + cranial radiation) and maintenance CHOP, result in a 79% CR and 49% DFS rate at nine years [107].

In a retrospective analysis of 80 patients, the chemotherapy protocol utilized did not influence CR rate, CR duration, or overall survival [108]. Presence of B-symptoms, increased LDH level, age over 40 years, failure to achieve CR, and advanced stage were found to be factors associated with a poorer outcome [101,108,109]. The disseminated nature of the disease combined with frequent marrow relapses implies little role for mediastinal radiotherapy as part of curative treatment. In a recent study, excellent results were noted following a shorter duration of treatment without maintenance therapy achieved by incorporating intermediate- and high-dose cytarabine into the induction and consolidation regimens [110].

High recurrence rates despite initial complete responses have led to the evaluation of high-dose chemotherapy with stem cell support or allogeneic BMT in select patients with high-risk LBL [111–114]. To evaluate the impact of autologous BMT utilized as consolidation for patients achieving a complete remission, 21 consecutive patients were treated with autologous BMT with cyclophosphamide and TBI after achieving CR with an LSA2–L2-based regimen [111]. After a median follow-up of five years, 14 patients were alive and well, with an actuarial DFS of 66%. In a similar study, 6 of 9 poor-risk patients treated with autologous BMT following first remission were reported to be alive and disease free after a median follow-up of 4.5 years [112]. In a retrospective analysis by the European Group for Bone Marrow Transplantation (EBMT), of 105 patients who underwent high-dose therapy and autologous

BMT for LBL, the actuarial overall survival rate at six years for the entire group was 42% [113]. Disease status at BMT was the major determinant of outcome. Overall survival was 63% for patients transplanted in first CR, compared with 15% for those with resistant disease at the time of transplantation. Transplantation in second CR resulted in a 31% rate of actuarial overall survival at six years [113]. For patients transplanted in first CR, univariate analysis failed to identify any factors at presentation that predicted for outcome after transplantation [113]. Although these preliminary results suggest that high-dose chemoradiotherapy followed by autologous BMT may improve long-term DFS in advanced-stage adult lymphoblastic lymphoma, it is unclear whether the currently available results reflect a selection bias or an actual improvement in DFS rates over intensive chemotherapy regimens in the absence of randomized clinical trials. Data are inadequate regarding the role of allogeneic BMT in patients with LBL. The results obtained with allogeneic BMT performed in a limited number of patients who have an HLA-identical sibling are comparable to the results obtained with autologous transplantation [114].

Anaplastic large cell lymphoma (ALCL)

Anaplastic large cell lymphomas (ALCLs) compose 2.5% of the lymphomas [86]. These patients are characterized by frequent peripheral lymphadenopathy (>80%), extranodal disease (>40%), and skin involvement (20%). Marrow involvement is rare in ALCL cases (3%–7%). Two histologic subtypes of ALCL are recognized and are defined as *common* (ALCL-CT) and *Hodgkin's-like* (ALCL-HL) types. The Hodgkin's-related ALCL cases almost always present with mediastinal involvement (100%), have a higher frequency of bulky disease (57% vs. 24%) and present at a relatively younger age (median age 27 vs. 34) compared to the common type [115]. ALCL cells express the Ki-1 (CD30) marker, an antigen expressed by activated B- and T-cells, embryonal carcinoma cells, and Reed–Sternberg cells. Up to 70% of ALCL cases of T-cell derivation carry a translocation that results in the fusion of the NPM (nucleophosmin) gene on chromosome 5q35 with the novel ALK (anaplastic lymphoma kinase) gene on chromosome 2p23 [116–118]. Serum levels of the soluble form of the CD25 and CD30 molecules can be used as a marker at diagnosis or relapse [119,120]. Most patients present with stage III/IV disease. Stage is highly predictive of achieving a CR and disease-free or overall survival. Localized skin lesions have an excellent prognosis, and occasional spontaneous regressions are noted [121].

ALCL responds to CHOP and other third-generation chemotherapy regimens in a manner similar to the other aggressive lymphomas in terms of response and relapse-free survival rates [115,122–124]. In patients treated with CHOP with or without radiotherapy, a 52% five-year survival was obtained [123]. Advanced stage, bulky disease, and B symptoms adversely affect overall prognosis [122,124]. Third-generation regimens have also been utilized with

excellent results (overall survival 73%), without a significant difference between the two histologic subgroups [122]. While the Southwest Oncology Group had not noted a difference in overall and event-free survival between ALCL and other aggressive lymphomas, the French retrospective analysis of 146 ALCL cases did demonstrate the diagnosis of ALCL as an independent favorable prognostic factor [125–127]. The detection of the associated NPM/ALK hybrid gene product (p80) appears to correlate with outcome [128]. The five-year survival rate for 30 of 105 patients with positive p80 staining was significantly higher than for those patients lacking the marker (79% vs. 32%). However, none of the p80+ cases coexpressed B-cell antigens, and the median age for this group was significantly lower than the p80− group (16 vs. 55 years) [128]. These findings indicate the heterogeneity in Ki+ anaplastic lymphomas and suggest that these patients may fall into biologically distinct groups [129].

Autologous BMT has been utilized, in part because of the low frequency of marrow involvement in ALCL. In a single-institution study combining the F-MACHOP regimen and autologous BMT following a conditioning regimen of cytarabine, etoposide, cyclophosphamide, and carmustine (BAVC), an actuarial disease-free and overall survival of 100% was obtained at four years [130,131]. This promising report suggests that sequential intensive treatment followed by radiation therapy and high-dose chemotherapy with stem cell support may prevent early relapses.

Peripheral T-cell lymphomas

The peripheral T-cell lymphomas (PTCLs) are characterized by a mature T-cell phenotype combined with considerable morphologic heterogeneity [132,133] and compose 30% of the aggressive lymphomas [19] and 7% of all the lymphomas [86]. PTCL subcategories include immunoblastic lymphoma, anaplastic lymphoma, and diffuse mixed lymphoma [19]. These lymphomas tend to present in advanced stages with B-symptoms, and have more frequent splenic and skin involvement [19,134]. A retrospective analysis of 168 PTCL patients demonstrated a mean age of 52.9 years, a low incidence of bulky disease (11.3%), a 32% incidence of a mediastinal mass, and a disease-free survival of 38.2%, supporting the subclassification of T-cell lymphomas proposed by the REAL schema [135].

The French investigators have confirmed the higher relapse rates and poorer prognosis associated with PTCLs reported earlier [19]. One hundred and eight patients with PTCL received an aggressive sequential regimen (LNH-84) composed of three or four courses of doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisolone (ACVB); consolidation with high-dose methotrexate, ifosfamide, etoposide, asparaginase, and cytarabine; and a randomized late intensification with two courses of cytarabine, cyclophosphamide, teniposide, bleomycin, and prednisone (AraCVmB). The observed recurrence rate of 43% was significantly higher than reported in other

aggressive B-cell lymphomas [19]. Although the PTCLs have different clinical characteristics and poorer outcome with conventional or intensive combination chemotherapy, specific treatment strategies for this entity have not yet been defined. Low DFS rates observed in PTCLs justify enrolling these patients into investigational protocols.

Data on allogeneic or autologous BMT in PTCLs is very limited at present. In a recently reported study, five patients treated with allogeneic (1) or autologous (4) BMT were alive and disease free at a median follow-up of two years, suggesting the feasibility of this approach in younger patients with a poor prognostic index [136].

New drugs in the management of the aggressive lymphomas: Retinoic acid

Certain retinoic acid derivatives (N-4-hydroxyphenyl-retinamide, all-trans-retinoic acid) can induce apoptosis or growth inhibition in T-cell lymphoma cell lines [137–139]. Retinoic acid derivatives have been used in patients with low-grade lymphomas (CTCLs), with a 45% overall response rate when administered as a single agent [140]. Five of 12 patients with peripheral T-cell lymphoma were reported to have a complete response to 13-*cis* retinoic acid therapy, whereas no evidence of response was observed in patients with B-cell lymphoma [141]. In a recent study reported from China, 3 of 5 patients with ALCL, 4 of whom relapsed after intensive chemotherapy, achieved a prolonged CR after being treated with 13-*cis* retinoic acid [142]. The median survival of the whole group has not yet been reached at a median follow-up of 40 months. According to the preliminary in vitro and in vivo data available, 13-*cis* retinoic acid appears to be a promising agent for certain lymphomas and merits further investigation.

Immunotherapy for aggressive lymphomas

Monoclonal antibodies

Monoclonal antibodies directed against antigens expressed on the surface of lymphoma cells have been designed in an effort to mediate complement and antibody-dependent cell-mediated cytotoxicity. These agents may also have direct antiproliferative effects. Initial studies performed with mouse monoclonal pan-B-cell (anti-CD20) and pan-lymphoid (Campath) antibodies have documented temporary and partial responses [143,144]. Humanized monoclonal anti-CD20 (IDEC-C2B8) has been used in patients with recurrent low-grade NHL. Of 37 patients treated with the IDEC-C2B8 antibody, clinical remissions were observed in 46% of patients (3 CRs and 14 PRs). The onset of tumor response ranged from 1 to 4 months posttreatment. The median time to progression was reported to be 10.2 months, with five patients exceeding 20 months [145]. None of the patients with aggressive histology responded to

therapy in this trial. Although there are few clinical data on the use of IDEC-C2B8 in patients with aggressive histology, several clinical trials have been initiated. ECOG currently is testing CHOP and IDEC-C2B8 versus CHOP alone in elderly patients with aggressive histology.

Radioimmunotherapy

Radioimmunoconjugate therapy represents a new treatment modality for patients with relapsed NHL. Radioimmunoconjugates may overcome the limitations of unconjugated monoclonal antibodies. The dose-limiting toxicity of this approach appears to be myelosuppression. Nonmyeloablative low-dose regimens consisting of repeated administrations of the radiolabeled antibody are capable of inducing objective responses in up to 70%–80% (30%–50% CR) of patients, with an average median duration response of a year in complete responders [146–148]. In one trial, ^{131}I-radiolabeled anti-CD20 antibody was reported to be associated with a high rate of durable remissions exceeding 16 months in patients with refractory B-cell lymphoma [148]. Although the majority of treated patients had low-grade histology, a 60% response rate was noted in the 15 patients with aggressive histology (4 complete and 5 partial remissions) [148]. Patients receiving high-dose radioimmunotherapy (25–30Gy) consisting of ^{131}I-radiolabeled anti-CD20 antibody coupled with stem cell support can achieve CR rates up to 85%, with a progression-free survival of 62% and an overall survival of 93% at two years [149,150]. In this study, 3 of 4 patients with aggressive histology achieved a complete remission [150].

Immunotoxins

Saporin, a toxin that inhibits protein synthesis by inactivating ribosomes, has been conjugated to anti-CD30 antibodies. This immunotoxin has been successfully used to treat SCID-mice bearing ALCL, with a 30% complete response rate [151]. The combination of this BerH2(anti-CD30)-saporin conjugate along with anti-CD19 monoclonal antibody appears to be promising in studies of SCID-mice bearing human ALCL [152]. IL-2 fusion toxins have been used in patients with cutaneous T-cell lymphomas [153]. The Anti-Tac(Fv)-PE-38 immunotoxin is currently being tested in cutaneous T-cell lymphomas in a phase-I clinical trial by the NCI. The B4-blocked ricin immunotoxin, directed against the CD19 antigen, has been tested in patients with HIV-associated lymphoma [154], following autologous transplantation, and in some patients with aggressive lymphoma [155,156].

Vaccine therapy

Vaccination with tumor-associated protein antigens may induce objective responses in patients with recurrent lymphoma. Idiotype-based vaccination has

been used in patients with B-cell NHL. In these studies, tumor-specific anti-idiotype (anti-Id) monoclonal antibodies were used to induce humoral and proliferative responses in tumor-specific cytotoxic T-lymphocyte precursors (CTLp). With this approach, 8 of 11 patients with low-grade B-cell NHL have been reported to achieve either a complete response or stable minimal disease [157]. This treatment approach has not yet been applied to aggressive lymphomas. Autologous dendritic cells pulsed ex vivo with tumor-specific idiotype protein to stimulate host antitumor immunity has induced complete responses in lymphoma patients when infused as a vaccine [158]. Treatment of B-cell tumors using idiotypic vaccination is a promising new approach in the management of lymphomas and requires additional investigation [158,159].

Adoptive immunotherapy

Adoptive immunotherapy will likely be incorporated into the autologous or allogeneic stem cell transplant setting of the future. Adoptive cellular therapy with donor-derived EBV-specific cytotoxic T cells has been used in the management of children with EBV-induced aggressive lymphomas occurring after a T-cell depleted allogeneic BMT [158–162]. These approaches may offer new treatment options for aggressive opportunistic lymphomas and could also be applied in the setting of minimal residual disease following conventional or high-dose therapy.

Conclusion

Standard chemotherapy cures only a subgroup of patients with aggressive lymphoma. Patients with low-risk features are more likely to be cured with standard therapy. High-risk patients require alternative strategies if a meaningful long-term survival is to be achieved. Clinical investigation into the use of high-dose therapy is essential, and new agents or new modalities should be offered routinely to patients with high-risk disease. A risk-adapted approach to treatment for the aggressive lymphomas is strongly recommended, especially in the setting of a clinical trial.

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4. The molecular biology of acute promyelocytic leukemia

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In this chapter, our goal is to review and summarize current knowledge of the molecular biology of acute promyelocytic leukemia (APL). The cure of up to 75% of patients with APL [1,2] represents a remarkable and fascinating success story in medicine, and the treatment of APL with retinoic acid serves as a paradigm for the use of differentiation therapy in other types of human cancers. The high cure rate of APL is due in part to the extreme sensitivity of leukemic promyelocytes to the differentiating effects of the vitamin A derivative all-*trans* retinoic acid (ATRA), and it thus becomes critical to delineate the mechanisms by which APL cells respond to this and other retinoids. As discussed in detail below, the defining molecular aberration in APL is disruption of the alpha receptor for retinoic acid, RARα, and its reciprocal in-frame fusion with one of four partner genes (Table 1). As will become clear in this chapter, these fusion proteins, while they lack classic transforming activity, nevertheless have the capacity to disrupt hematopoiesis and effectively inactivate the molecular switch that governs promyelocytic maturation. Our focus here will thus be on the structure and function of the four fusion genes involved in APL pathogenesis, and on the mechanisms by which APL cells are sensitive to, and in some cases become resistant to, retinoids. It is hoped that a full understanding of the structure and function of these fusion molecules will provide a conceptual framework for a fuller understanding of the molecular signals that control both normal and malignant myelopoiesis. It is further hoped that this type of understanding will lead to the development of novel therapeutic strategies for other subtypes of acute myeloid leukemia which, unlike APL, remain largely resistant to conventional or differentiation therapy.

Although the clinical syndrome of APL was originally described in the 1950s [3], the molecular era of APL biology began in 1977, with the observation that essentially all APL cases contained a reciprocal translocation between chromosomes 15 and 17 [4]. The unique sensitivity of myeloid cell lines and leukemic promyelocytes to retinoic acid [5,6], coupled with the subsequent localization of the retinoic acid receptor (RAR) gene to chromosome 17q [7,8], led investigators to hypothesize that RAR might be involved in APL pathogenesis. Indeed, this hypothesis was quickly proven true, as several

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Table 1. Translocations and fusion genes involved in APL

Karyotype	Fusion gene	Frequency[a]	Responsive to ATRA	Reciprocal product[b]	Refs.
t(15;17)(q22;q21)	PML-RARα	>99.9% of cases	Yes	Yes, in 75%	9–12; 104–106
t(11;17)(q23;q21)	PLZF-RARα	7 cases	No	Yes	40, 41
t(5;17)(q32;q21)	NPM-RARα	1 case[c]	Yes[d]	Yes	112, 181
t(11;17)(q13;q21)	NuMA-RARα	1 case	Yes	No	113, 182

[a] Percentage of all cases or number of published cases.
[b] Reciprocal fusion transcript detected by RT-PCR.
[c] An additional case of atypical APL with an apparent t(5;17) has been described [215], but without formal proof of involvement of NPM.
[d] Clinical response difficult to judge, but cells are responsive to ATRA in vitro.

independent groups demonstrated rearrangement of the RAR gene in most if not all APL cases [9–11]. It was subsequently shown that the RARα locus was fused to a gene at 15q22, now termed PML, resulting in the production of two novel genes, PML-RARα and RARα-PML (reviewed in [12]). It is now known that a small fraction of patients with otherwise classic APL will lack the t(15;17) due to submicroscopic PML-RARα translocations [13,14]; in these cases, rearrangement of the PML and RARα genes can be confirmed by either fluorescence in situ hybridization (FISH) or Southern blotting, and the PML-RARα transcript can be detected using reverse-transcription polymerase chain reaction (RT-PCR). An even smaller fraction of APL cases have one of three described variant translocations, each of which also involves rearrangement of RARα (Table 1; discussed in detail below). Thus, the primary and defining molecular event in APL is the translocation of the RARA locus at 17q21 to one of four alternative genomic loci, and it can now be stated that in the absence of cytogenetic or molecular evidence of RARα rearrangement, the diagnosis of APL is untenable. Because PML-RARα is involved in 99.9% of APL cases, the majority of this chapter will center on the function of this fusion protein and on the structure/function of the two normal proteins, PML and RARα, of which it is composed. However, significant attention will also be given to the other three gene fusions since, despite their rarity, an understanding of their function may offer significant further insight into the molecular pathogenesis of APL. Finally, we will address the use of molecular methods for the diagnosis of APL and for detection of minimal residual disease.

Structure and function of normal PML

Structural features

The PML gene belongs to a large gene family that has a variety of associated functions, most related to DNA replication–repair and transcriptional regula-

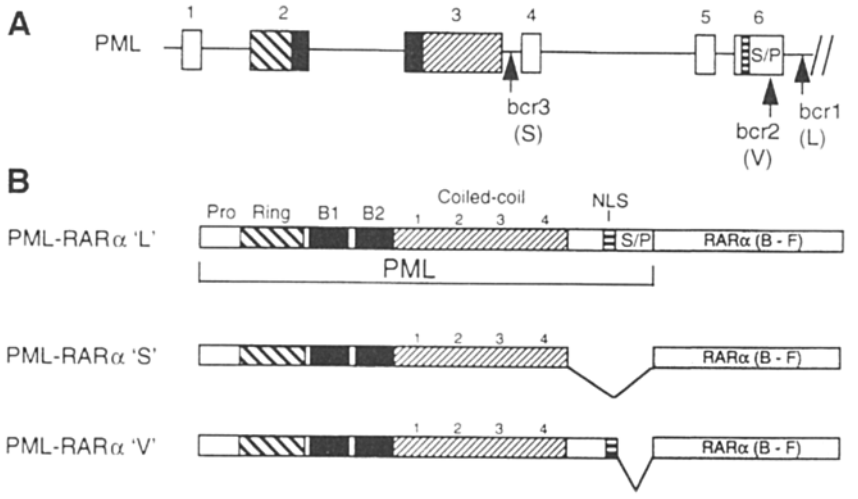


Figure 1. Breakpoints in the PML gene and PML-RARα isoforms involved in APL. (**A**) Schematic diagram of the 5′ region of the PML gene showing the three major breakpoint regions (bcrs) involved in formation of the chimeric PML-RARα fusion gene. PML exons (rectangles) are numbered. Breakage of PML in one of the three bcrs gives rise to distinct PML-RARα molecular isoforms, as shown in B. (**B**) Schematic drawing of the PML-RARα L, S, and V isoforms, created by fusion of the RARα B through F domains with PML exon 6 (L, V isoforms) or PML exon 3 (S isoform). Motifs in the PML gene common to each isoform include a proline-rich amino terminus (Pro), a Ring finger (Ring), two so-called B-boxes (B1 and B2), and a coiled-coil or leucine zipper domain consisting of four distinct coils. Domains B through F of RARα are present in all isoforms. Note that a putative nuclear localization signal (NLS) and a serine/proline-rich region (S/P), located in PML exon 6, are not present in the PML-RARα S isoform, and that the V isoform lacks variable amounts of the serine/proline-rich region of PML exon 6. See text for further discussion.

tion [15–18]. The core region of the encoded PML protein has a characteristic tripartite structure consisting of a characteristic cysteine/histidine-rich ($Cys_3HisCys_4$) cluster called the RING motif, followed by two alternative cysteine/histidine clusters called B-boxes, followed, in turn, by an α-helical coiled-coil domain (Figure 1) [19]. This tripartite structure is shared by eight other genes of this protein subfamily, two members of which, in addition to PML — the ret finger protein [20] and T18/TIF1 [21] — can form oncogenic hybrid proteins as the result of tumor-associated chromosome translocations. All three cysteine/histidine clusters have heavy metal binding potential, but only the RING finger motif has been shown to bind zinc ions and, in response, to modify its conformation [19]. Although many zinc finger proteins function by binding to specific nucleotide sequences in DNA, PML has not been shown to bind DNA, and current evidence indicates that the RING finger domain functions through protein–protein interactions. These interactions are likely facilitated by hydrophobic amino acid heptad repeats in the coiled-coil region, which serves as a critical interface for the formation of PML homodimers and of heterodimers with PML-RARα in APL cells [17,22,23].

The core region of PML is preceded at the amino-terminus by an acidic proline-rich region, a feature of many transcriptional activators, and it is succeeded at the carboxy terminus by a serine/proline-rich region, which is highly variable due to alternative splicing of the last six exons of the gene transcript [15–17,24]. The carboxy terminus contains many potential phosphorylation sites, including a single casein kinase site that is notable because its position at the beginning of exon 7 is conserved in all 13 known alternatively spliced mRNA isoforms [24]. Phosphoamino acid analysis indicates that multiple serine residues and at least one tyrosine residue are phosphorylated in native PML protein [25].

Cellular expression

PML is expressed in a wide variety of cell types, in most at low levels [26–28]. Its expression is increased in actively proliferating benign or malignant cells either in vivo or in vitro at least partly due to upregulation during the late G1/early S phase of the cell cycle [25–27]. Another major factor that can affect PML expression is the presence of certain inflammatory cytokines, particularly interferons [27–31]. These cytokines are related to the direct activation of interferon-specific response elements in the promoter region of the PML gene that results in rapid upregulation of PML mRNA [30,32]. This activation apparently accounts for the increased expression of PML in areas of inflammation, particularly in selected cells such as endothelial cells and activated macrophages [27,28]. Some differences in the array of PML mRNA isoforms were observed in granulocytes and monocytes following interferon treatment, suggesting possible differential isoform function [31].

A notable feature of PML expression is its localization to the nucleus, where it is associated with organelles called nuclear bodies (NBs) that form part of the nuclear matrix [17,25,33–35]. In proliferating cell lines in vitro, typically 10–20 PML NBs are present per cell nucleus, which appear as discrete dots by immunofluorescent staining of the associated PML protein, and as dense fibrillar spheroids by electron microscopic examination. However, the number, form, and size of NBs are quite variable, perhaps due to differences in cell growth, differentiation, or activation state. Based on an analysis of these morphological variations, it has been suggested that the NBs may provide a storage receptacle for PML, which can be released in an unaggregated, active form at certain times, e.g., during the early S phase of the cell cycle [26,36]. A recent study indicates that a larger fraction of PML than previously thought is present in the nucleoplasm and can be recruited onto nuclear matrix-associated NBs, e.g., by arsenic trioxide (As_2O_3), where it becomes susceptible to proteolytic degradation [37]. By immunocytochemical methods, PML NBs have been discriminated from alternative NBs that house enzyme activities involved in pre-mRNA splicing and DNA replication [35]. At least five other proteins have been shown to colocalize to PML NBs [30,34,35,38,39]. Intriguingly, one of these is PLZF (promyelocytic leukemia zinc finger), which is

involved, like PML, in the formation of an alternative hybrid protein with RARα (PLZF-RARα) in rare cases of APL [40,41]. The additional demonstration of a direct interaction between PML and PLZF suggests a possible cross-regulatory function [39]. Another of the PML-associated proteins, PIC1, has a ubiquitin-like domain that could be involved in proteolysis of NB-associated PML [38]. More generally, all the PML NB-associated proteins are coordinately regulated in response to interferon and/or by cellular infection by certain DNA viruses, suggesting that one function of the PML NB complex is related to host–virus interactions [26].

Functional activity

Tests of PML functional activity in vitro indicate that it has potent growth-inhibitory activity in a variety of cell types, including in the APL cell line NB4 [26,42,43]. Additionally, it has been shown to inhibit the transforming activity of selected oncogenes and to suppress the transcriptional activity of some gene promoters [42]. These functional activities have further been demonstrated to be dependent on the integrity of the RING and dimerization domains, as well as a nuclear localizing region, all of which are essential for localization of the PML protein to NBs [19,44]. Preliminary data suggest that the transcriptional repressor activity of PML is functionally conserved within the Ring/B-box/coiled-coil family of proteins and is mediated by the coiled-coil domain [45]. Taken together, these results indicate that PML is a transcriptional repressor with a marked negative effect on cell growth. It is intriguing to speculate that, in PML-RARα-positive APL, PML is targeted (via the RARα DNA binding domain) to the promoters of retinoic acid-regulated genes. It may be further hypothesized that the presence of PML suppresses expression of such genes, either directly through its own repressor function, or indirectly through the recruitment of alternative transcriptional corepressors.

Structure–function of normal RARα

Relation to nuclear hormone receptor gene superfamily

RARα is a member of the steroid/thyroid hormone receptor gene superfamily, which encode proteins that function as *trans*-acting, ligand-dependent regulators of gene transcription (reviewed in [46]). In order to perform this common function, these proteins contain two essential domains, namely, a DNA binding domain (DBD) near the amino terminus and a ligand binding domain (LBD) near the carboxy terminus (Figure 2). The DBD, which contains two characteristic zinc finger motifs, serves to bind the proteins to specific oligonucleotide sequences, namely, hormone response elements (HREs), in the promoter region of select genes, many of which have central effects on cell and tissue growth, differentiation, and homeostasis. The RARs, along with the

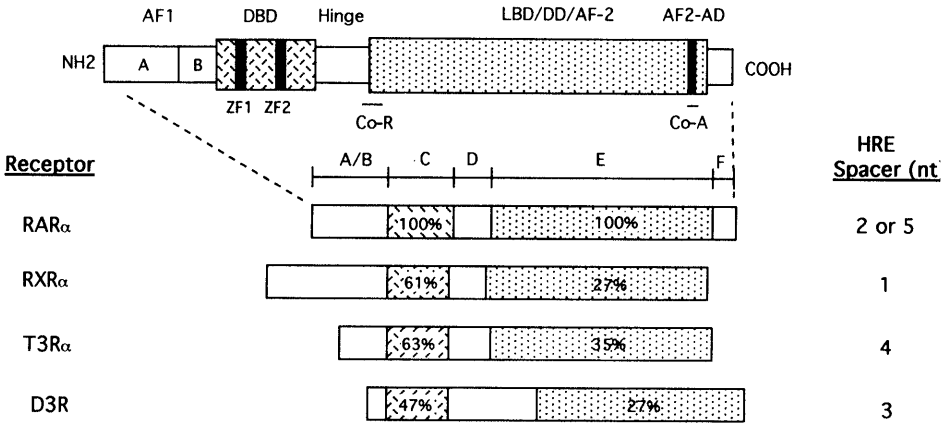


Figure 2. Structure of RARα and its homology to related members of the RAR–RXR–T3R–D3R branch of the steroid–thyroid hormone receptor gene superfamily. AF1, activation 1 (ligand-independent) domain; DBD, DNA binding domain; LBD/DD/AF2, overlapping ligand binding, dimerization, and activator function 2 (ligand-dependent) domains; ZF1 and ZF2, zinc fingers 1 and 2; AF2-AD/Co-A, AF2 activation core domain containing consensus sequence for binding coactivators (Co-A); Co-R, corepressor binding region. A–F indicate normally recognized regions/domains of these proteins. Percentage numbers indicate the degree of amino acid sequence identity of the DNA binding (C regions) and ligand binding (E regions) of each receptor protein to RARα. HRE, hormone response element; the numbers beneath indicate the number of nucleotides (nt) between the two one-half site direct repeats, PuG(G/T)TCA.

thyroid hormone receptors and vitamin D_3 receptor, belong to one major branch of the steroid hormone receptor superfamily, members of which have the common property of binding to HREs consisting of two direct repeats of the hexanucleotide PuG(G/T)CTA (Figures 2 and 3A). These receptors also share the property of binding to HREs as a heterodimer with common, adaptor proteins called retinoid X receptors (RXRs; Figure 3A). The discriminator for HRE specificity is the number of nucleotides *between* the two direct repeats, which is two or five for retinoic acid response elements (RAREs), three for vitamin D_3 RE (VDRE) and four for thyroid hormone RE (TREs). Additionally, HRE spacers with one nucleotide have specificity for RXR homodimers or, in some cases, may heterodimerize with RAR in reverse polarity, i.e., with the RAR in the 5′ RARE half-site rather than in the usual 3′ RARE half-site relative to RXR, as shown in Figure 2 and as required for transactivational activity [47].

Related to their common HRE binding sequence, these receptor proteins maintain a high degree of evolutionary conservation of the DBDs/C regions (≥50%) (Figure 2). A lesser but significant degree of conserved homology is observed in the LBD/E regions. Since the chemical structure of the cognate ligands is quite different, much of this retained homology can be attributed to additional common functional activities (see below). There is little

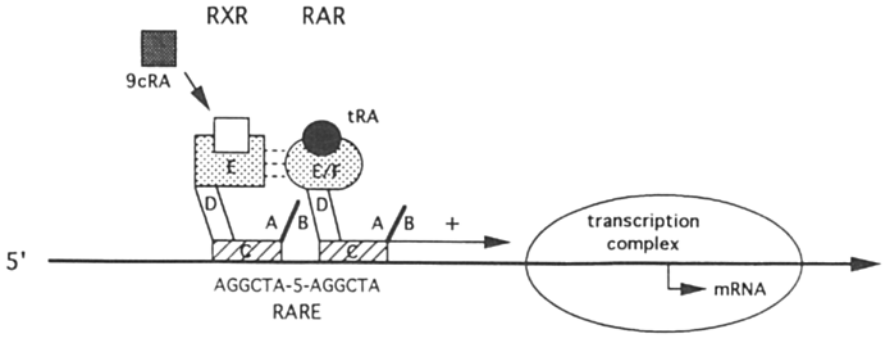
interreceptor homology between the A/B or D regions, and the F region is confined to the RARs. The A region, which is the deleted portion of RARα in the PML-RARα fusion protein (see below), contains autonomous, ligand-independent transcriptional activation function (AF-1), which was recently demonstrated to be regulated by phosphorylation of a specific serine residue [48,49]. The D region serves a rotational or 'hinge' function related to heterodimer formation on HREs (schematically shown for RAR in Figure 3A) and contains an incompletely conserved sequence at the E-region boundary important for interaction with a repressor protein [47]. The function of the F region is unknown.

Multiple functions of the E-region/LBD

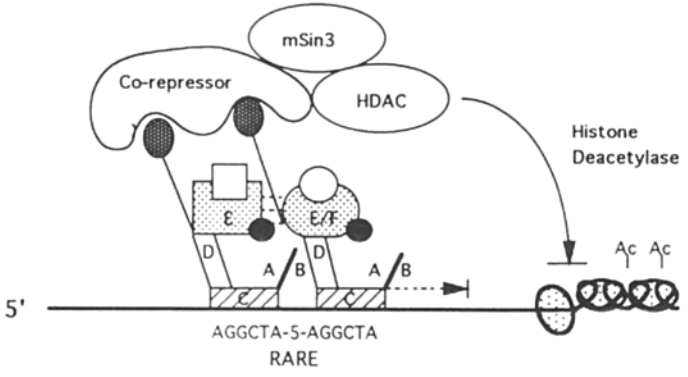
The E region of retinoid receptors has multiple functions, which have been analyzed by many methods, including crystallographic analysis of purified LBD in the presence and absence of ligand (ATRA) [46,50]. Such analysis has defined the three-dimensional structure of the region to consist of 12 α-helices and 2 β-strands linked by a series of angled loops. From this conformational model, it has been determined that key amino acids from many components of the LBD contribute to the formation of a binding pocket for ATRA, while those from a more restricted area stabilize the RAR:RXR dimerization interface through specific noncovalent interactions. The presence of ATRA in the binding pocket produces an overall tightening of the three-dimensional structure, a very prominent feature of which is a closing of the 12th, carboxy-terminal α-helix over the opening to the occupied binding site. The latter movement (schematically illustrated in Figures 3B and 3C), which has been likened to the springing of a mousetrap, has been correlated with the acquisition of the capacity of a highly conserved seven-amino-acid-long stretch in α-helix 12, called the AF-2 activation domain (AF-2 AD), to interact with crucial coactivator (Co-A) proteins (Figure 3C). This interaction is mandatory for activation of the transcription complex, since mutations that prevent AF-2 AD/Co-A interactions completely eliminate transcriptional activation by RARE-bound RARs [46,51]. Recently, it was further demonstrated, following ATRA-binding to RARα, that the RXR component of the RAR-RXR heterodimer can bind its specific ligand (9-*cis* RA) and that a conformational change in the AF-2 AD region of both receptors acts synergistically in the activation response [52]. Another consequence of the ligand-induced conformational change of the LBD of RARs is ejection of corepressor (Co-R) proteins from binding sites in the hinge-proximal LBD region of the RAR component of the RARE-bound RXR:RAR dimer [47,53]. The binding of these corepressors in the absence of ligand is functionally active, reducing promoter-driven transcription below basal levels (Figure 3B).

Recently, it was discovered that both corepressors and coactivators interact through specific consensus amino acid sequences with yet other nuclear proteins that can enzymatically decrease or increase the level of histone

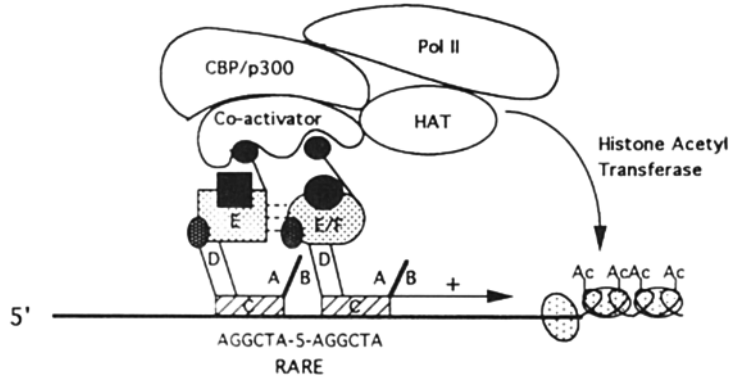
A. RAR-RXR Dimer on RARE



B. Co-Repressor Complex



C. Co-Activator Complex



acetylation in chromatin, an important determinant of the availability of genomic DNA for transcriptional activity (schematically illustrated in Figures 3B and 3C; see [54,55] for minireviews and references). Other common themes that have emerged from investigations of several RAR-interactive transcriptional intermediary factors (TIFs) is that they are shared by alternative transcriptional regulatory systems and that competition for limiting amounts of these factors can produce intersystem effects that broaden the integrated response of cells to a stimulus primarily directed at one transcriptional regulatory system [46,56,57].

Heterogeneity of RAR expression

Although the above account focuses on the common elements of RAR function, the RAR system appears to have been selected for its capacity to generate a great heterogeneity of responses needed to produce specific regulatory effects in different cell/tissue types at different stages of differentiation/development [46]. Several levels of molecular heterogeneity have been identified that contribute to this flexibility of function. There are three independent RAR genes (α, β, and γ) and three independent RXR genes (α, β, and γ). Each of these genes has at least two different isoforms due to alternative 5′ exon selection during pre-mRNA processing, which results in alternative A/B re-

Figure 3. Model for regulation of an RA-responsive gene promoter. (**A**) Formation of RAR-RXR heterodimer on retinoic acid response element (RARE). The diagram shows RXR bound to the upstream RARE half-site and RAR bound to the downstream half-site, the two separated by a five-nucleotide spacer and with noncovalent bonding (dotted lines) between apposed portions of the E domains of the two proteins. ATRA has already bound to RAR, which produces steric changes in RXR, which then becomes permissive for binding its specific ligand 9-*cis* RA. (**B**) Formation of repressor complex in the absence of ligand binding. Specific peptide motifs in the proximal portion of the E/LBD-region and distal D/hinge-region (Co-R box) interact with complementary motifs in nuclear receptor corepressor proteins (N-CoR or SMRT), which, in turn, bind to a common adapator protein mSin3 that forms a complex with further interacting proteins with histone deacetylase activity (HDAC). Removal of negatively charged acetyl residues from core histones leads to tighter interaction of these proteins with the negatively charged DNA helix producing condensed heterochromatin, blocking transcriptional activity. (**C**) Formation of activator complex. Ligand binding produces a conformational shift in the RAR and RXR proteins, such that the twelfth, carboxy-terminal α-helix swings over the bound ligand. This shift helps to discharge corepressor proteins and brings peptide motifs in the AF-2 activator domain (AF-2 AD) core sequence (Co-A) into contact with complementary motifs in coactivator proteins, which, in turn, interact with other proteins, including a CREB-binding protein (CBP/p300) group. CBP contains target sites for many transcriptional regulatory signals and, additionally, interacts with other proteins that have histone acetyl transferase activity and proteins of the basal transcriptional apparatus, including RNA polymerase II. The acetylation of core histones loosens the chromatin structure, allowing access of transcription factors to promoter sites and fostering transcriptional activity.

gions attached to a common C to F region. These considerations alone provide 64 different combinations. Additional recognized sources of heterogeneity, each of which can modify selective target gene transcriptional activity, include interaction with alternative active retinoids, e.g., 9-*cis* RA, the unique natural ligand for RXRs that can bind to RARs equally well as ATRA; interaction with different RAREs, which may have 1, 2, or 5 nucleotide spacers and can have either a G or T residue in the third half-site position (Figure 2); and variable expression of different TIFs in specific cell types. This incomplete list, as well as quantitative variations in the RAR-associated cofactors and more distant elements affecting chromatin accessibility, provides a source of potential diversity that is extraordinarily complex [46].

In individual cell types, the expression spectrum of RAR-related molecules appears to be much more limited. Thus, RARα and RXRα are the predominant receptors expressed in normal and leukemic myeloid cells [58–60]. The preeminent role of RARα in the response of myeloid cells to retinoic acid has been reinforced by studies with mutants of RARα that abolish AF-2 AD function, which can arrest multipotential normal hematopoietic cells at the promyelocyte stage of differentiation [61] and which are associated with the loss of terminal differentiation response of a myeloid leukemia cell line (HL-60) to retinoic acid [62,63].

PML-RARα: structure, function, and role in the pathogenesis of APL

Breakpoint regions in PML and RARα

The salient structural features of the PML-RARα fusion gene are shown in Figure 1. Most notable is the presence of three distinct PML breakpoint cluster regions (bcrs), one located in the 5′ region (bcr-3, breakpoint in PML intron 3), and two located more distally, either in PML exon 6 (bcr-2) or intron 6 (bcr-1). As opposed to the variable breakpoints seen in PML, the genomic breakpoints of the RARα gene cluster within a single 20-kb span between the second and third exons [64]. Identification of the PML breakpoint is readily achieved using RT-PCR with flanking primers from PML exon 3 and RARα exon 3 or 4. It is apparent from Figure 1 that each breakpoint gives rise to a distinct PML-RARα mRNA molecule or isoform, and these isoforms have respectively been designated S, V, and L for Short (PML intron 3 or bcr-3 breakpoint), Variable (PML exon 6 or bcr-2 break), and Long (PML intron 6 or bcr-1 break). Each of the isoforms contains at a minimum exons 1–3 of PML and exons 3–9 of RARα, in a fusion that preserves the open reading frame; thus, the motifs critical for the oncogenic function of PML-RARα include 1) the PML proline-rich, RING, B-box, and coiled-coil domains, and 2) domains B through F of RARα, which include the DNA binding, ligand binding, and heterodimerization motifs of this molecule. The PML-RARα V and L isoforms also contain additional PML coding sequence, as shown in Figure 1.

The L-type PML-RARα isoform is expressed in approximately 55% of adult patients with APL, while the S and V isoforms are expressed in approximately 35% and 8% of patients, respectively [65]. In the pediatric population, the V isoform accounts for a larger proportion of cases than in adults (27% in the study by Kane et al. [66]), with a corresponding decrease in the number of S-form cases [66].

Structural and functional differences between PML-RARα isoforms, and prognostic significance of isoform type

There are several structural differences between the PML-RARα S, V, and L isoforms. A major potential structural motif missing in the S isoform, but present in both the L and V isoforms, is a putative nuclear localization signal (NLS) located in the proximal region of PML exon 6. In addition, a serine-proline rich region, located in distal PML exon 6, is absent in all S isoform and some V isoform cases [67,68]. The importance of these and other structural elements to the subcellular location and function of the native PML protein has been examined in 3T3 cells [44]. In that system, a mutant PML protein that lacked the NLS was found in the cytoplasm, did not participate in nuclear body formation, and was functionally inactive [44]. In contrast, a mutant PML protein that lacked the serine–proline-rich region was found in the nucleus in normal-appearing nuclear bodies and was fully functional [44]. Although some nuclear localization must be provided by the NLS in the RARα moiety, it is possible that the PML-RARα S isoform is less able to enter the nucleus than the L isoform and is therefore less able to respond to ATRA. Although this hypothesis is supported by immunofluorescence data [17], a recent report [69] suggests that the type S PML-RARα isoform, at least when bound to ATRA, is found exclusively in high-molecular-weight nuclear complexes. It is interesting to note that, while the L isoform can apparently bind ATRA as a monomer, the S isoform cannot, and appears to bind ATRA only as part of a larger protein–protein complex that is contained in the nuclear fraction [69].

In a large multi-institutional study involving 230 patients with molecular and clinical data, type S (bcr-3) APL patients were noted to present with higher absolute white blood cell (WBC) and blast counts compared to type L (bcr-1) patients [65]. Furthermore, type S patients have also been reported to have an increased incidence of secondary chromosomal abnormalities [70]. These somewhat subtle clinical differences have been difficult to reproduce in vitro, since only minimal differences have been observed in the ATRA-dependent transcriptional activity of the S or L isoforms in cotransfection assays; furthermore, when cultured in vitro, blasts from patients with type S and type L APL were reported to differentiate to a similar degree in response to ATRA [68]. However, a recent report [69] has suggested that the PML-RARα L and S isoforms differ in both specificity and affinity of binding to ATRA and 9-*cis* RA. In particular, the S (bcr-3) isoform was reported to have a lower binding affinity for ATRA but a higher binding affinity and specificity

for 9-*cis* RA, compared to the L isoform [69]. This increase in 9-*cis* RA binding affinity of the S isoform translated into an enhanced ability of 9-*cis* RA to activate a reporter gene (TRE-tk-Luciferase) containing a retinoic acid response element (RARE) in the presence of the type S compared to the type L PML-RARα isoform, and also to an increased sensitivity of type S (compared to L) APL blasts to differentiation in response to 9-*cis* RA [69]. It is also worth noting that blasts from at least some V-isoform patients were reported to respond poorly to ATRA in vitro [68], and this rare patient subgroup (approximately 8% of all adult APL patients) may have a slightly worse prognosis than type S or type L patients [67]. Finally, when both the S and L isoforms were expressed in TF1 cells (a human erythroleukemia cell line), significant differences were noted between the isoforms in their ability to transduce a retinoid-mediated signal [71]. Despite these somewhat subtle biologic differences noted in vitro, and despite earlier reports [72,73] that S isoform patients had poor outcomes, more recent studies suggest that isoform type is not an independent determinant of outcome in patients who are treated with standard ATRA/chemotherapy regimens [65,74]. Thus, it seems likely that appropriate combination differentiation/cytotoxic therapy overcomes any negative prognostic influence accorded by isoform type.

Subcellular localization of PML-RARα

As assessed by immunofluorescence staining, PML-RARα is located primarily in the nucleus in hundreds of small ($\leq 0.1\,\mu m$), poorly characterized structures termed *microspeckles* [33–35,75] that are associated with the nuclear matrix [76]. The protein composition of these microspeckles is not completely known, but RXRα [35], the primary biliary cirrhosis autoantigen Sp100 [77], PML itself [33–35], and PLZF [39] colocalize with PML-RARα to these structures. These morphologic results have led to the suggestion that PML-RARα physically sequesters wild-type PML and RXRα, and perhaps other proteins, into nonfunctional structures, and thereby interferes with normal PML and/or RXRα function in a dominant-negative manner. The distinct granular or microparticulate staining pattern that is seen in NB4 or APL cells with anti-PML and anti-RARα antibodies is considered diagnostic of APL [78]. The functional significance of PML-RARα-induced PML relocalization to nuclear microspeckles is unknown, since a PML-RARα mutant that failed to direct such relocalization nevertheless retained full biologic activity in a differentiation inhibition assay [23]. Subsequent studies have suggested that PML-RARα localization to nuclear microspeckles is necessary, but not sufficient, for biologic activity [76].

Interactions of PML-RARα with itself and other proteins

Both the PML and RARα moieties of PML-RARα contribute a dimerization domain to the fusion molecule (Figure 4), suggesting that PML-RARα will

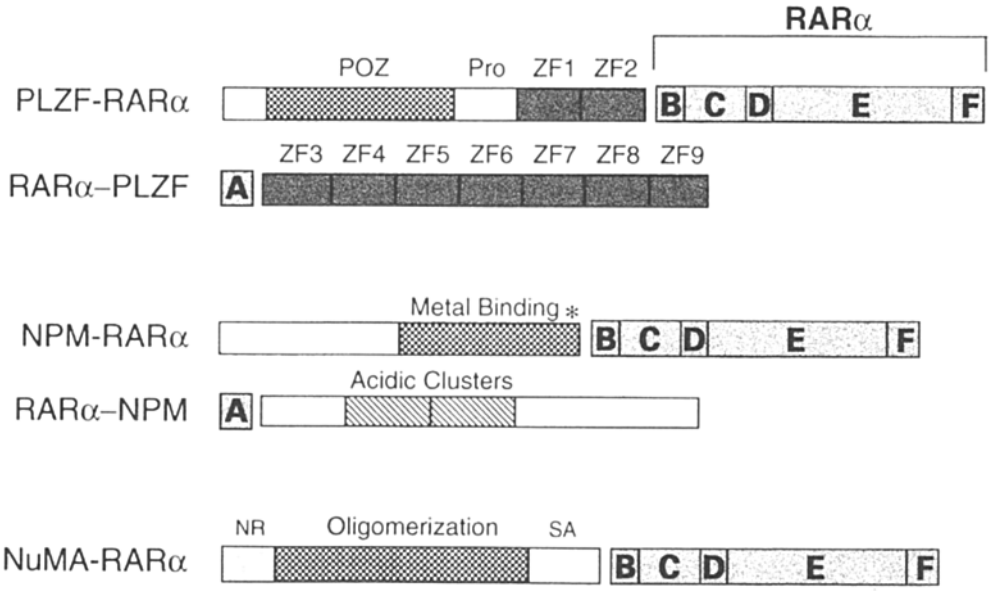


Figure 4. Structure of rare RARα fusion variants that also cause an APL phenotype in vivo. In this highly schematic diagram, the major structural features of each protein are shown as discrete rectangles. See text for full discussion of the putative function of these various domains. Abbreviations: POZ, POx-virus and Zinc domain; Pro, proline-rich; ZF, zinc finger; NR, nuclear relocalization motif in NuMA; SA, spindle assembly motif in NuMA. *, phosphorylation site in NPM. The reciprocal fusion protein is also shown, except for RARα-NuMA, since this fusion product was not detected in the single case reported. Domains of RARα are shown in bold upper case letters.

interact with a variety of different proteins in vivo. Such proteins are strong candidates for involvement in the oncogenic potential of PML-RARα, since deletion of the entire dimerization interface of PML blocks the antidifferentiative function of PML-RARα in vitro [23], and since this domain appears to possess transcriptional repressor activity [45]. PML-RARα has been shown to homodimerize with itself and to heterodimerize with both PML and RXRα [17,22,34,79]. The interaction between PML and PML-RARα has been demonstrated in vivo using coimmunoprecipitation assays, immunofluorescence analysis, and the yeast two-hybird system [17,22,34,35], and appears to be mediated by the second of four heptad repeats in the coiled-coil domain [23]. Interestingly, however, a PML-RARα mutant containing a deletion of the second heptad of the dimerization interface, which abolishes heterodimerization of PML-RARα with PML (but not PML-RARα homodimerization), was fully active in the differentiation inhibition assay, suggesting that PML/PML-RARα interactions are not critical for the differentiation block [23].

Heterodimers between PML-RARα and RXRα are mediated by the

heterodimerization domain of the RARα moiety [22,79,80], and direct functional interference with RXR-mediated signaling has been reported [22], at least in vitro. Furthermore, as discussed below some RXR-mediated signals are transduced poorly in NB4 cells [81], leading to the hypothesis that PML-RARα may block myeloid differentiation via sequestration and functional inactivation of RXRα. There is, in fact, evidence that titration of RXR can block myeloid differentiation both in vitro and in vivo. In murine bone marrow cells, for example, forced overexpression of RARα blocks myeloid development at the promyelocyte stage [82], presumably by titrating out RXRα and thereby preventing an RXRα-dependent differentiation response. In vitro, overexpressed RARα can block differentiation of hematopoietic cell lines [23,83], an effect that is reversed by forced expression of RXRα [23]. Finally, targeted expression of a dominant negative RXRβ to myeloid cells in transgenic mice led, in some mice, to a differentiation block at the promyelocyte stage [84]. It would appear from these data that a signal transduced via RXR homo- or heterodimers may be necessary for myeloid cells to mature past the promyelocyte stage. However, once again the significance of this interaction in the overall function of PML-RARα is unclear, since forced overexpression of RXRα did not relieve the differentiation block imposed by PML-RARα in vitro [23]. Finally, it should be noted that PML-RARα, like RARα, binds the nuclear receptor corepressor SMRT in vitro [85]; like RARα, this binding is inhibited in the presence of ATRA and is mediated by a conserved N-Co-R box motif in the RARα moiety [85]. The functional consequences of this binding and its relevance to APL remain to be determined.

DNA binding and transcriptional activity

The RARα DNA binding domain (DBD) in the PML-RARα molecule is functionally intact and mediates binding of PML-RARα to consensus retinoic acid response elements (RAREs) in vitro. Deletion of this domain blocks retinoid-induced transactivation of an RARE by PML-RARα and partially impairs the ability of PML-RARα to block differentiation in U937 cells [23], indicating that DNA binding by PML-RARα (through the RARα DBD) is critical for its antidifferentiative (and possibly oncogenic) activity. In addition, the RARα DBD is necessary for the cell death response that is observed upon PML-RARα expression in most hematopoietic and nonhematopoietic cell lines in vitro [76]. PML-RARα can bind DNA as a homodimer (homodimerization being mediated by the coiled coil domain of PML [79]) or as a multimeric complex in association with RXRα [22,79], and there are both similarities and differences in the specificity of binding to different RAREs between PML-RARα homodimers and RAR/RXR heterodimers [22,79]. The combined data suggest that the biologic activity of PML-RARα depends on targeting this molecule to retinoic acid regulated genes in vivo, with a consequent (presumably inhibitory) effect on expression of such genes. As discussed

earlier, it is possible that the RARα moiety of PML-RARα targets PML, a known transcriptional repressor, to RA-regulated genes in vivo and results in their transcriptional suppression. If correct, this model suggests that identification of RA target genes that are silenced or otherwise modulated by PML-RARα will be a critical next step in understanding the pathogenesis of APL.

PML-RARα: in vitro functional studies

Most nonhematopoietic and hematopoietic cell lines are nonpermissive for PML-RARα expression. In one study [76], inducible expression of PML-RARα could be achieved in only two nonhematopoietic cell lines (NIH3T3 mouse fibroblasts and U2OS osteosarcoma cells); similar finding were seen in hematopoietic cell lines, where expression could be achieved in only 4 of 10 lines tested (KG1a, U937, K562, and TF1 [76]). Induction of PML-RARα expression in either NIH3T3 or U2OS cell lines resulted in accelerated cell death, whereas its expression in 'permissive' hematopoietic cell lines led to cell death inhibition [71,86–88]. These cell type-specific differences in PML-RARα biologic activity in vitro remain unexplained, but the ability to express PML-RARα in at least some hematopoietic cell lines has allowed a limited functional analysis of this protein in vitro. Several early studies demonstrated that PML-RARα could inhibit myeloid differentiation in vitro [23,83,86,89,90]. For example, in U937 promonocytic cells, vitamin D_3- and TGFβ-induced differentiation, as measured by expression of the monocytic differentiation antigens CD11a, CD11c, CD14, and FcRI, was markedly inhibited in the presence of unliganded PML-RARα [86,90]. In a similar vein, transient expression of PML-RARα in HL-60 cells inhibited both granulocytic and monocytic differentiation in response to low doses of ATRA or vitamin D_3, respectively [89]. Finally, as discussed above, PML-RARα has been shown to increase cell survival in several hematopoietic cell lines when grown either in low serum [86] or in growth-factor-deficient medium [71,87,88]. These biologic activities of PML-RARα in vitro mimic to some extent the presumed function of PML-RARα in vivo; however, it is perhaps most remarkable that the major effect of PML-RARα expression in most cell lines is in fact cell death, perhaps related to the growth-suppressive effect of the PML moiety [76]. These results are in striking contrast to those seen with, e.g., BCR-ABL, which is directly transforming in mouse fibroblasts. At a minimum, such results suggest that the 'oncogenic' function of PML-RARα is extremely cell type specific and requires a cell type (presumably a myeloid precursor) that has a unique, very specialized cellular milieu.

PML-RARα: in vivo functional studies

In a chicken leukemia model [91], a retrovirus expressing a mutant form of PML-RARα (containing single amino acid changes in the RING and second B-box domains) transforms immature bone marrow cells and causes acute

leukemia when injected intravenously into chicken embryos. The leukemic cells do not resemble promyelocytes, suggesting that the retrovirus transforms an earlier hematopoietic progenitor than that affected by PML-RARα in humans. It is also notable that the chicken bone marrow cells transformed by mutant PML-RARα do not differentiate, or undergo PML-relocalization, in response to ATRA, even though the mutations do not abrogate the ability of the mutant PML-RARα molecules to transactivate the βRARE in cotransfection assays in vitro [91]. Thus, whether the lack of response to ATRA is directly attributable to the mutations themselves or to inherent differences between the chicken and human systems is not clear. However, it is possible that the mutations prevent PML-RARα degradation in response to ATRA [92,93], which would account for the lack of PML redistribution and perhaps as well for the lack of effect of ATRA on differentiation. Similar PML mutations might be speculated to result in ATRA resistance in vivo [94]; however, to date, no such mutations have been found in either retinoid-sensitive or resistant human APL cases [95].

PML-RARα has now been expressed in transgenic mice under the control of three different promoters. When expressed in myeloid cells with the CD11b promoter, which is most active in mature myelomonocytic cells, PML-RARα caused a subtle impairment of myelopoiesis characterized by decreased clonal growth response of peripheral blood cells to GM-CSF [96]. Despite over two years of observation, leukemia did not develop in these mice; in fact, myeloid cells expressing PML-RARα appeared more susceptible to radiation-induced cell death, since sublethal irradiation led to more profound and long-lasting cytopenias in transgenic as compared to wild-type mice [96]. These results offer support for the model discussed above, in which the function of PML-RARα (pro- or antiapoptotic) is critically dependent on the cell type in which it is expressed. It is also worth noting in this regard that attempts to express PML-RARα in transgenic mice under the control of ubiquitous promoters (e.g., β-actin) have failed, presumably due to the toxic effect of PML-RARα in embryonic cells.

The hMRP8 promoter, which is active throughout myeloid development [97], has also been used to drive expression of PML-RARα in transgenic mice [98]. The authors describe a preleukemic phenotype characterized by subtle impairment of myeloid maturation, which progresses to acute leukemia in a small percentage of mice following a latency period of 3 to 9 months. Although leukemia developed in less than 5% of the PML-RARα-positive mice, it was similar in many respects to human APL in that most of the leukemic cells were promyelocytes, a bleeding diathesis developed in some mice, and, most importantly, the leukemia cells underwent apparent differentiation in response to ATRA [98]. Finally, two groups have used a genomic fragment from the promyelocyte specific human cathepsin G gene to drive PML-RARα expression in transgenic mice [99,100]. Both groups observed a preleukemic, myeloproliferative phase characterized by an accumulation of mature myeloid cells in both the bone marrow and spleen. After a latency period of six months

to a year, from 10% [100] to 30% [99] of the transgenic mice developed acute leukemia. In both reports, malignant splenocytes or bone marrow cells were sensitive to ATRA in vitro, although there was a difference as to whether ATRA induced apoptosis [99] or differentiation [100] of the leukemic cells.

Although none of these in vivo models perfectly recapitulates human APL, several important conclusions can be drawn. First, these studies conclusively demonstrate the potential of PML-RARα to disrupt hematopoiesis in vivo, and offer strong support for the premise that PML-RARα is integrally involved in the pathogenesis of human APL. Second, and perhaps most surprisingly, each of the studies in which acute leukemia developed reported its development only after a prolonged latency period that lasted from 6 to 12 months; furthermore, only a minority of mice developed leukemia, even after this long incubation period. These results suggest either that PML-RARα alone is insufficient to cause APL (i.e., additional genetic changes are necessary) or that the expression vectors used to date do not target PML-RARα to the appropriate cell type in vivo. Additional genetic changes, in additional to PML-RARα, that may contribute to the development of APL are unknown, but obvious candidates are the reciprocal fusion protein RARα-PML or the truncated PML protein(s) described by Pandolfi et al. [101]. At least one group has generated transgenic mice expressing both PML-RARα and RARα-PML, and preliminary results suggest that such mice have an increased penetrance of the APL phenotype [102]. It is notable in this regard that one case of apparent true APL has been reported that lacks the PML-RARα fusion (at both the DNA and RNA level) but has the RARα-PML genomic fusion and expresses RARα-PML transcripts [14]. Finally, as discussed above, the stage of myeloid development at which PML-RARα is expressed appears to be crucial in determination of the resultant phenotype Particularly intriguing is the difference between the myeloproliferative phenotype observed when PML-RARα is driven by the cathepsin G promoter, and the comparable preleukemic phenotype in MRP8-PML-RARα transgenics, which is myelodysplastic in nature (and the minimal phenotype in CD11b-PML-RARα transgenics). These studies further corroborate the usefulness of ATRA in APL and promise to provide valuable in vivo models to both understand the mechanisms by which ATRA works in APL and to test alternative retinoids or other agents (e.g., arsenic) for their utility in the treatment of APL.

Reciprocal product RARα-PML

Sequence analysis of the t(15;17) breakpoints indicates that this translocation occurs without significant deletion of either the RARα or PML genes [103]. This finding raises the possibility that the reciprocal RARα-PML fusion gene could play a supplementary role to the PML-RARα fusion gene in the pathogenesis of APL. However, the information content of RARα-PML is rather limited, consisting of the A region of RARα that has ligand-independent

modulatory effects on the transcriptional regulatory activity of RARα, a short common region of PML encoded by exon 7 that contains a unique casein kinase-II site, and a variety of serine–proline-rich carboxy-terminal segments in different transcripts due to variable PML exon splicing (Figure 1). Also, RARα-PML is expressed in only about 75% of PML-RARα-positive cases [104–106]. Nevertheless, the possibility of a functional role has been suggested because 1) the hybrid mRNA generated by RARα-PML encodes an open translational reading frame for the production of a full-length protein in all RARα-PML-positive cases; 2) one APL case has been described that expressed RARα-PML mRNA in the absence of PML-RARα mRNA [14]; and 3) coexpression of RARα-PML with PML-RARα in transgenic mice appears to increase the penetrance of APL [102]. Despite these considerations, the clinical importance of RARα-PML expression remains unclear. In a recent study of a large number of uniformly treated APL patients, no association of the presence or absence of RARα-PML with either APL cell sensitivity to ATRA-induced differentiation in vitro or clinical outcome after induction therapy with either ATRA or chemotherapy in vivo could be demonstrated [106]. This study additionally demonstrated that two forms of RARα-PML, RARα1-PML and RARα2-PML, are present in 28% of APL cases due to alternative use of 5′-end exons, providing different RARα A regions. In all L- and V-form PML-RARα-positive cases, this produces a translational frame shift to encode only a short RARα2 peptide. Variations in the expression of RARα2, like those for RARα1-PML, had no significant biological correlates, but, interestingly, it was found that expression of RARα2-PML was linked to RARα1-PML expression [106], whereas normal RARα1 and RARα2 gene promoters have been reported to be independently regulated [107]. This finding could reflect some epigenetic difference between the translocated and normal RARα alleles that could have an impact on other processes, such as susceptibility to mutation or responsiveness to transcriptional regulators.

The molecular basis of the retinoid response in APL cells

Introduction

Treatment with ATRA alone induces transient complete remission in the vast majority of patients with APL, and it is well established that this reduction of leukemic burden occurs via induction of differentiation of the malignant clone [108–110]. It is not known whether this effect of ATRA is general (i.e., whether retinoic acid is required for nonmalignant myeloid progenitors to mature beyond the promyelocytic stage) or whether the effect of ATRA is specific for promyelocytes expressing PML-RARα or another APL-specific RARα fusion. In support of the latter concept is the observation that retinoic acid has little or no effect on differentiation of other types of AML blasts, as well as the fact that mice lacking a functional RARα gene have no demon-

strable defects in myeloid maturation [111]. In addition, it is well established [65] that cytogenetic or molecular documentation of the t(15;17) predicts response to ATRA clinically. Thus, the preponderance of evidence suggests that the PML-RARα molecule itself mediates ATRA sensitivity. It is notable that 2 of the 3 rare cytogenetic subtypes of APL, namely, NPM-RARα and NuMA-RARα, also appear to respond to ATRA [112,113], whereas patients (and cells) with PLZF-RARα do not [41,114], at least at the doses currently used. What makes some molecular types of APL sensitive to ATRA, and what is the molecular basis of the remarkable sensitivity of most APL cells to retinoids? Complete answers to these questions are not yet available, but there is a considerable amount of experimental data that addresses these issues, as discussed below.

In vitro studies with the cell line NB4

The NB4 cell line, originally derived from a multiply relapsed and retinoid-resistant APL patient [115], represents the best available in vitro model of APL; it contains the t(15;17), and expresses PML-RARα (L isoform). Treatment of NB4 cells for 96 hours with ATRA at a concentration of 10^{-8}M or higher results in a significant inhibition of cell proliferation and, ultimately, growth arrest [116–118]. This outcome is accompanied by typical morphologic changes of myeloid differentiation, by the expression of differentiation markers [118,119], and by the ability to reduce nitroblue tetrazolium [120]. After 6–8 days of retinoid treatment, NB4 cells undergo DNA fragmentation and apoptosis [117]. Interestingly, the growth inhibition, differentiation, and apoptotic response can be achieved with retinoids that have only RARα agonist activity [117,118], suggesting that these effects require retinoid binding and activation of either the native RARα molecule or the RARα moiety of PML-RARα. Recent work [121] strongly suggests that it is retinoid binding to the RARα moiety of PML-RARα that mediates the differentiation and apoptotic response, since an NB4 subclone with a mutation in the ligand binding domain of PML-RARα (which was shown to not bind ATRA) failed to differentiate in response to ATRA. In that clone, wild-type RARα (expressed from the normal allele) was intact as assessed by Western blot and RA binding studies [121]. Binding of ATRA (or RARα-selective retinoids) to PML-RARα therefore appears to activate differentiation and ultimately apoptosis in APL cells.

Studies in cell lines engineered to overexpress PML-RARα

Stable expression of PML-RARα in 'permissive' hematopoietic cell lines has been shown to increase the sensitivity of such cell lines to retinoids, an effect not seen in identical cell lines that express PLZF-RARα [122]. For example, treatment of U937 cells containing PML-RARα with 10^{-6}M ATRA inhibited cell proliferation and accelerated differentiation [86]. When PML-RARα was

expressed in either lymphoid [123] or erythroid [71] cell lines, it mediated a strong, retinoid-dependent, apoptotic response. Thus, PML-RARα can transduce a retinoid-induced differentiative/apoptotic signal in cells of diverse hematopoietic lineages; although the relevance of this finding to APL is unknown, the inherent growth-suppressive and apoptotic properties of PML-RARα in many different cell types [76] suggest that it may mediate a similar signal in vivo in primary APL blasts that are exposed to pharmacologic concentrations of ATRA.

The potential role of bcl-2

Retinoic acid treatment of both NB4 cells [120] and APL blasts [124] results in a significant reduction in expression of the antiapoptotic protein bcl-2. This downregulation of bcl-2 by retinoic acid is tightly coupled to differentiation, but not apoptosis, of these PML-RARα-positive cells [120,124]. Although bcl-2 can be downregulated by retinoic acid in non-APL cells [125,126], there is some indication that APL blasts or PML-RARα-expressing cells may be particularly susceptible to bcl-2 downmodulation by retinoids [71,124]. While it is possible that loss of bcl-2 is simply a consequence of differentiation of APL cells (and thus not a primary effect of retinoids), its downregulation may nevertheless be critically involved in the cell death response of APL cells to retinoid/chemotherapy combinations, as now used clinically [1,2]. Since bcl-2 can protect cells from chemotherapy-induced apoptosis [127], the decline in bcl-2 levels in response to ATRA in APL blasts (presumably associated with differentiation of those cells) may sensitize these cells to the apoptotic effects of chemotherapy [126,128]. This finding alone may explain the remarkable clinical results seen with ATRA/chemotherapy combinations in APL, and has prompted study of ATRA and/or alternative methods of bcl-2 modulation in non-APL cells [128,129]. Indeed, preliminary reports suggest that ATRA/chemotherapy combinations may have utility in other types of myeloid malignancies [130].

Other examples of ATRA-regulated gene expression in PML-RARα-expressing cells

Experiments performed in parallel with retinoid-sensitive (parental) and retinoid-resistant NB4 cell lines may offer clues to PML-RARα-specific regulation of gene expression. In this context, *retinoid-resistant* denotes an NB4 subclone, generally selected after prolonged culture in ATRA-containing medium, that fails to differentiate in response to ATRA or other retinoids. In such resistant cell lines, PML-RARα is either presumed or proven to be nonfunctional [81,121,131–133], whereas native RARα is generally preserved. Of particular interest are genes induced in the parental NB4 line but not in resistant subclones, since these genes may be involved in specific differentiative or apoptotic programs that are activated specifically by ligand-

activated PML-RARα. A candidate gene is transglutaminase II (TG-II), a cytosolic protein cross-linking enzyme that is known to be regulated by retinoids in human leukemia cell lines [134]. TG-II mRNA is induced by ATRA in parental NB4 cells but not in an ATRA-resistant (PML-RARα-deficient) cell line [81]. That TG-II is induced by ATRA preferentially in PML-RARα-expressing cells was further demonstrated by showing induction of TG-II by ATRA in U937 cells expressing PML-RARα, but not in control U937 cells, and by demonstrating an increase in TG-II enzymatic activity in response to ATRA in APL blasts, but not in blasts from two patients lacking PML-RARα [81]. Thus, as concluded by Benedetti et al. [81], TG-II gene expression is likely to be mediated through a PML-RARα signaling pathway. The role of TG-II in myeloid differentiation and/or apoptosis remains unclear, although it has been associated with apoptosis in many systems [135]. In HL-60 cells, for example, TG-II is upregulated by retinoids, primarily via an RXRα-mediated pathway, and has been considered to be involved in the RXR-mediated apoptotic response of these cells [60]. In NB4 cells, both TG-II mRNA and activity are induced well before any signs of apoptosis are evident, suggesting that TG-II induction may be related more to differentiation than to apoptosis in the NB4 system [81].

Evidence that ATRA treatment of PML-RARα-expressing cells activates RXR-mediated gene expression programs

In NB4 cells, RXR-selective agonists fail to induce expression of either the CD18 or the TG-II genes [81], whereas both ATRA and 9-*cis*-RA are strong inducers of these genes. In retinoid-resistant (PML-RARα-defective) NB4 cells, by contrast, CD18 is induced by RXR-selective agents [81]. Furthermore, TG-II is strongly induced by RXR-selective agonists in HL-60 cells. This result implies that at least some RXR-mediated responses are deficient in NB4 cells, consistent with a dominant-negative inhibition (and possibly sequestration) of RXRα by PML-RARα (see, e.g., [22]). Additional support of this hypothesis derives from the observation that NB4 cells differentiate to mature monocytic cells only if cultured in the presence of both vitamin D_3 and ATRA [90]. This result implies either synergistic activation of two different signaling pathways (unlikely, since either agent alone causes no monocytic differentiation) or, more likely, a 'release' of active RXR that is then able to heterodimerize with the vitamin D receptor and activate expression of the monocytic gene program. Two mechanisms may account for this effect. In one scenario, ATRA binding to the RARα moiety of PML-RARα degrades PML-RARα [92,93], and frees RXR to transduce a retinoid-mediated signal (or to cooperate with other nuclear hormone receptors). Alternatively, binding of ATRA to PML-RARα may relieve an allosteric inhibition of RXRα function, as occurs for native RAR/RXR heterodimers [52]. However, the impact of sequestration and functional inactivation of RXRα in APL pathogenesis is at present difficult to judge, since RXRα itself is downregulated upon treatment

of NB4 cells with ATRA [119] and, perhaps more importantly, forcec overexpression of RXRα fails to reverse the inhibition of differentiation con ferred by PML-RARα in response to vitamin D_3 and TGFβ [23].

Other genes modulated by ATRA in APL cells

Recently, additional genes have been identified that may be specifically in duced by retinoids in cells that express PML-RARα. *JEM-1* is a transcribec locus at 1q24 that was cloned by differential display from NB4 cells treated fo 48 hours with ATRA [136]; the Jem-1 cDNA encodes a putative 45-kD protein with homology to b-ZIP transcription factors such as Jun and Fos. It expression is not myeloid restricted, but it appears to be retinoid-inducible only in NB4 cells that contain functional PML-RARα [136]. The *RIG-E* gene [137], also isolated by differential display from ATRA-treated NB4 cells, is a fairly widely expressed gene with homology to the LY-6 family of stem cel antigens defined in mice. It is induced by ATRA in both NB4 and HL-60 cells [137], and thus its retinoid-induced transcription may be mediated by norma RARα/RXRα. Finally, Yu et al. [138] recently reported the cloning of a gene termed *RIG-G* (*R*etinoic acid *I*nduced *G*ene-G). *RIG-G* is homologous tc some members of the Interferon Sensitive Gene (ISG) family, and indeed is itself induced in NB4 cells by α-IFN as well as ATRA [138]. *RIG-G* was not induced by ATRA in HL-60 cells or in an ATRA-sensitive breast carcinoma cell line, suggesting that its induction may be relatively specific to cells express ing PML-RARα. There are apparently at least two other members of the ISG family that are induced by ATRA in NB4 cells (discussed in [138]), suggesting a significant degree of cross-talk between retinoids and interferons in myeloid differentiation of NB4 cells [31,139].

Nuclear reorganization and PML-RARα degradation in response to ATRA

Treatment of NB4 cells or APL blasts with ATRA leads to disappearance of nuclear microspeckles and to their replacement by a limited number of larger, spherical nuclear dots that stain with antibodies directed against PML and other components of the nuclear body [33–35,75]. These dots have been variously called nuclear bodies (NBs) or PML oncogenic domains (PODs). Although it was originally suggested that these NBs contained PML-RARα [35,75], with the implication that ATRA caused a nuclear redistribution of PML-RARα, subsequent work [33,34,37] suggests that only a very limited amount of PML-RARα is relocalized to NBs in response to ATRA; instead, PML-RARα-specific immunofluorescence, as assessed using RARα antibodies, appears to decline after ATRA treatment, suggesting a direct effect of ATRA on PML-RARα protein levels. Indeed, several groups have recently shown that ATRA can cause specific degradation of PML-RARα, as assessed by Western blotting [79,92,93]. Presumably, this loss of PML-RARα protein upon ATRA treatment allows PML and other proteins normally associated

with the NB to reaggregate into normal structures. This reorganization of the nuclear body structure, either as a consequence of or in association with PML-RARα degradation, may be a critical step in the differentiation process of APL cells. This concept is supported by data showing that retinoid-resistant NB4 subclones fail to reorganize nuclear body structure in response to ATRA [34,140], although the differentiation block (and recovery of normal nuclear body morphology) can be overcome by sequential treatment of at least some resistant cell lines with ATRA and cAMP [140]. Thus, there appears to be a fairly strict correlation between the acquisition of a differentiated phenotype and the recovery of normal nuclear body morphology.

Molecular aspects of retinoic acid resistance

Pharmacologic versus cellular basis of RA resistance

As described elsewhere in this book, clinical resistance to ATRA inevitably develops in all APL patients continuously treated with ATRA as a single agent (reviewed in [94,141]). There are two broad possible mechanisms by which this clinical resistance might develop: 1) decreased delivery of free retinoid to nuclear retinoid receptor sites where changes in gene transcriptional activity are made that overcome pathologically blocked leukemic cell differentiation or 2) alterations of the nuclear molecular response mechanism that prevent a cellular response despite the delivery of sufficient retinoid. The first mechanism refers to any alteration in the pharmacological processes involved in the retinoid delivery system, including gastrointestinal absorption, intravascular distribution, penetration into the extravascular pericellular fluid/matrix, and penetration into and distribution in the leukemic cell. This section will focus on the second broad mechanism, although some overall background considerations are made based on the more convenient division between pre-APL cell pharmacologic mechanisms and intrinsic APL cell mechanisms. This division is conceptually important, since much of the current information about molecular mechanisms of retinoid resistance is derived from leukemic cell lines, which may or may not be clinically relevant.

The early assessment of clinical retinoid resistance was that it was derived predominantly, if not entirely, from pharmacologic mechanisms rather than APL cellular/molecular mechanisms [94]. One plausible reason for this assessment was that the virtually universal development of retinoid resistance in a relatively short period of time in ATRA-treated APL patients (weeks to a few months) seemed more likely due to a common regulatory mechanism rather than stable molecular alterations in APL cells. In support of this hypothesis, pharmacokinetic data were developed that indicated that treatment with ATRA induces a systemic autocatabolic response that reduces achievable ATRA plasma levels by more than one half within a few days of the start of continuous oral administration [142]. Furthermore, it was demonstrated that

ATRA administration is associated with upregulation of a cytoplasmic ATRA binding protein (cellular retinoic acid binding protein-II; CRABP-II) in bone marrow [143] and skin cells [144] (and would presumably be upregulated in other cell types if tested). Since binding of CRABP-II enhances the oxidative metabolism of ATRA (reviewed in [145]), this systemic phenomenon could limit ATRA availability at critical APL cell nuclear sites.

There are, however, serious reasons for questioning the adequacy of the pharmacologic explanation for clinical retinoid resistance. Some of these reasons are, themselves, pharmacologic in nature and relate to the dichotomous observations that there are wide interpatient variations in plasma pharmacokinetics at first dose or after continuous administration [94,146], but that virtually all APL patients who do not succumb to early disease or toxic complications achieve complete remission (CR) on ATRA induction therapy [2,72,94,141,147,148]. Also, an alternative retinoic acid, 9-*cis* RA, which is not nearly as strongly subject to induction of autocatabolism as ATRA, and does not efficiently bind to CRABP-II, was not generally effective in patients with clinical ATRA resistance [149]. A more compelling reason, however, is the finding that the leukemic cells from many APL patients who relapse from ATRA therapy manifest resistance to ATRA as measured by decreased induction of differentiation in vitro [147,149–151]. This APL cellular resistance has been attributed to a quasi-pharmacological mechanism: increased expression of CRABP-II in the APL cells, which was demonstrated in four APL relapse cases, whereas ATRA-naive APL cells were reported not to express detectable levels of CRABP-II activity [141,150]. These relapse cases had terminated ATRA therapy up to 10 months before, suggesting that the increased CRABP-II ATRA binding activity was a stable trait. However, this study has not been confirmed, and apparently conflicting information has begun to appear, including a preliminary report that CRABP-II mRNA is constitutively expressed in most, possibly all, pretreatment APL cells [152] and findings that CRABP ATRA binding activity is expressed in ATRA-naive APL cells [69] (D.C. Zhou and R.E. Gallagher, unpublished). Also notable is that the model APL cell line NB4, which was derived from a clinically ATRA-resistant patient, expresses CRABP-II activity but remains sensitive to ATRA-induced differentiation [115,143]. Unfortunately, no detailed studies of ATRA oxidative metabolism in ATRA-sensitive versus ATRA-resistant cells have been performed to further evaluate the potential role of increased CRABP-II activity in diminishing access of free retinoic acid to the APL cell nucleus, although a study of APL cells from a small cohort of patients indicated that there is some interpatient variation in ATRA association with APL cells [153,154].

Retinoic acid resistance in myeloid leukemia cell lines

The finding that APL cells from clinically ATRA-resistant patients are intrinsically resistant to ATRA enhances the prospects that investigative findings

with ATRA-resistant cell lines will be clinically relevant. In this regard, two retinoic acid-sensitive myeloid leukemia cell lines have been extensively studied: HL-60, which has a predominantly promyelocytic phenotype but which lacks the t(15;17) [155], and NB4, which is t(15;17)-positive and expresses the PML-RARα fusion gene [115]. Although the NB4 cell line was derived from a patient clinically resistant to ATRA, extensive additional exposure to relatively high concentrations of ATRA (10^{-6}M) in vitro for a prolonged period (15 months) was required to select an ATRA-resistant subline called NB4-RA^r [156] or NB4-R1 [119]. During this long selection period, it was noted that only a subpopulation of NB4 cells was resistant to ATRA and that the resistance not only was reversible and but also differentially affected the sensitivity to retinoic acid-induced differentiation and apoptosis [120]. Similar subline instability was noted during the prolonged selection of an ATRA-resistant subline of HL-60 cells [157]. Notably, NB4-R1 cells remain partially sensitive to ATRA, since terminal differentiation can still be induced by agents that increase cellular cyclic AMP (cAMP) levels if the cells are first pretreated, i.e., primed, with ATRA [133]. Still further selection of NB4-R1 cells, however, with ATRA as well as a cAMP analogue yielded a subline, NB4-R2, which is completely resistant to ATRA-induced differentiation [133]. This series of experiments with NB4 cells importantly establishes that the development of leukemic cellular retinoid resistance can occur in multiple steps, which can be initially reversible, then dysregulatory, and finally aregulatory. In order to thoroughly understand the molecular basis of leukemic cell retinoid resistance, it would be of interest to study the cells in various stages of resistance development. However, doing so is experimentally difficult for the early stages of resistance and, thus, virtually all molecular studies of ATRA-resistance have been conducted on highly selected, stably resistant cell populations.

HL-60 cell retinoic acid resistance

We are aware of molecular mechanistic analyses of four independently-selected ATRA-resistant sublines or clones of HL-60 cells, all of which identified mutations in the primary ATRA target in myeloid cells, RARα ([62,63,158]; see also G. Brigati, personal communication). Further, all these mutations were located in the LBD. One missense mutation, which changed an amino acid from glycine to aspartic acid, was found in the relatively proximal part of the LBD (on a loop between α-helices 6 and 7; see [50]) and was associated with reduced RXR:RAR heterodimer formation on a 5-nucleotide spacer RARE [158]. Interestingly, this RA-resistant subline was found to have collateral sensitivity to differentiation induction by vitamin D_3, and it was speculated that the increased amount of RXR available from decreased binding to RARα might enhance heterodimerization of RXR with the vitamin D_3 nuclear receptor, increasing vitamin D_3 effectiveness [158]. Three of the mutations occurred in the AF-2 AD, and all of these altered the translational open reading frame such that truncated RARα proteins are encoded lacking part of

the critical coactivator binding consensus sequence (see Figures 2 and 3, [62,63], and G. Brigati, personal communication). Two of these mutations affect the same nucleic acid base, but one is heterozygous [62] and the other is homozygous [63]. This mutant protein was shown in transfection experiments to function as a dominant-negative inhibitor, i.e., to interfere with retinoid activation of a coexistent normal RAR by competition at the RARE DNA promoter-region binding site. Presumably, this is the endogenous mechanism of retinoid resistance in the heterozygotic RARα-mutant subline, i.e., interference with activation by the normal RARα allele product, but this cannot be the case in the homozygotic RARα-mutant subline, since HL-60 cells do not significantly express any other species of RAR, such as RAR-β or RAR-γ [60]. Thus, it is interesting that the heterozygous mutant subline is more resistant not only to retinoids but also to other differentiation inducers, such as dimethylsulfoxide and vitamin D_3, than the homozygous mutant subline [157,159,160].

NB4 cell retinoic acid resistance

In contrast to the HL-60 studies, molecular analyses of several highly-selected ATRA-resistant NB4 cell sublines or clones have implicated alterations in the PML-RARα fusion gene, while no abnormalities have so far been detected in the normal RARα gene [92,121,131,132,140]. This finding implies that, in true APL cells, PML-RARα rather than normal RARα is the dominant primary target for mediating the cellular response to ATRA. It also represents a unique potential mechanism for tumor cell progression, namely, secondary mutational change in a primary oncogenic product. For most ATRA-resistant NB4 sublines, the evidence for the nature of the alteration in PML-RARα is incomplete. One type of finding has been abnormal-sized PML-RARα protein bands on Western blot analysis that fail to react with antisera specific for the RARα carboxy terminus [131,140], suggesting that these may involve protein truncation mutations in the RARα region, possibly similar to those observed in RARα in most RA-resistant HL-60 cells. Another type of finding has been abnormal protein complexes identified after radiolabeled ATRA binding and gel chromatographic separation [81,121,132]. Considerable chromatographic heterogeneity of these ATRA-protein binding complexes was observed in comparison to the pattern of wild-type NB4 cells, implying that there could be a variety of abnormalities in PML-RARα or of the proteins interacting with PML-RARα. In one subline called R4, in which there was a lack of characteristic ATRA-bound PML-RARα protein complexes, a point mutation was detected in the LBD of the RARα region, while no mutations were detected in the RARα region of two other resistant sublines with abnormal complexes or in the normal RARα allele [121]. The R4 mutation resulted in a change from the amino acid leucine to proline in the eleventh α-helix, and it was postulated that this substitution might destabilize the helix function of the carboxy terminus of the RARα portion of the molecule essential for ATRA

binding (see above). The R4 PML-RARα mutant retained the ability to form heterodimers with RXR and to bind to RAREs. In transfection experiments, expression of the mutant protein produced constitutive repression of RARE-driven promoters in both the absence and presence of ATRA and in cotransfection experiments was associated with dominant negative repressor activity. In contrast to wild-type PML-RARα, the mutant protein was not degraded following ATRA exposure of R4 cells, suggesting either that this process requires initial activation of proteolytic activity by intact PML-RARα or that the mutation produces protective conformational changes in the mutant protein. Sequencing of the PML region of both the R4 mutant protein and the other NB4 cell ATRA-resistant sublines remains to be performed in order to determine if alterations of this region might also contribute to the altered binding complex behavior of these proteins compared to wild-type PML-RARα proteins. Recently, a second ATRA-resistant subline of NB4 cells was identified with a point mutation in the AF-2 domain of PML-RARα [161].

The heterogeneity of PML-RARα that has been observed in different RA-resistant NB4 sublines likely indicates a spectrum of molecular mechanisms that may be encountered in clinically relevant retinoid resistance and may provide insights into downstream consequences of alterations of the primary retinoic acid target and, also, of potential alterations of afferent modulators of PML-RARα activity. At least two RA-resistant NB4 cell lines with no readily apparent abnormality of PML-RARα can be at least partially resensitized to ATRA-induced cytodifferentiation by alternative agents known to have interactive effects with retinoids: the NB4-R1 subline, which can be 'rescued' with cAMP, as previously mentioned [119,133] and a spontaneously arising RA-resistant clone that can be 'rescued' with interferon-gamma [31]. Both these cofactors depended on retinoic acid for their full activity, suggesting that they either modify PML-RARα or some associated component of the transcriptional regulatory complex, such as a coactivator, and also suggesting that abnormalities of such cofactors could be a component of retinoid resistance in some instances. Abnormal phosphorylation is particularly suspect in the NB4-R1 subline, since this process could involve effects on direct cAMP-activated kinase activity [162] or indirect cAMP-regulated transcriptional regulation of specific phosphatase activities [163]. Perhaps related is the observation that following ATRA treatment, there is a marked decrease in a phosphorylated isoform of normal RARα in NB4 cells but not in NB4-R1 cells [119].

Several retinoid-modulated gene products are variably affected in different ATRA-resistant NB4 sublines. These products include PML-RARα itself, which, as noted above, is resistant to ATRA-induced degradation in the R4 mutant subline but is degraded in the NB4-R1 subline after ATRA exposure in the presence or absence of cAMP, i.e., with or without terminal differentiation [119]. Similarly, RXRα, which is rapidly downregulated by ATRA in wild-type NB4 cells and more slowly in the NB4-R1 subline [17,119,132], is constitutively expressed in other sublines with apparently defective PML-RARα function [119,132]. Also, in the NB4-R1 subline, RXRα levels are

preserved following ATRA-primed cAMP-induced differentiation [119]. Such observations, as well as the apparent constitutive expression of the normal RARα allele in APL cells following ATRA exposure, have led to the hypothesis that overcoming the inherent resistance of APL cells to physiologic levels of ATRA and of their acquired resistance to pharmacological levels of ATRA primarily relates to restoring a favorable balance of normal RAR:RXR heterodimer-promoted gene activity over abnormal PML-RARα:RXR heterodimer-/PML-RARα homodimer-promoted gene activity by disproportionately diminishing PML-RARα protein [92,119,132]. However, some published data are significantly at variance with the reported quantitative changes in PML-RARα, RXRα, and RARα in wild-type NB4 cells on which this hypothesis is founded [119,164,165]. Also, as discussed above, there is evidence that relief of sequestration of RXRα by degradation of excess PML-RARα is not restorative and that positive PML-RARα-specific activity is necessary to promote significant elements of the differentiative response of NB4 cells to retinoids [23,81,122]. Another important finding that contributes to the heterogeneity of retinoid-resistant NB4 sublines is that some retinoid-regulated genes, including CD18, CD38, and myeloblastin, remain inducible by ATRA in such cells [81,119,132]. This was the case even in the R4 subline that has constitutive PML-RARα repressor activity [121]. Thus, normal RAR:RXR transcriptional activation can occur on some gene promoters even in the presence of a dominant negative inhibitor. All the above findings and considerations lead to the general conclusion that heterogeneous defects can arise in NB4 cells selected for ATRA resistance in vitro at both the primary level affecting PML-RARα and at the abortive secondary response level, which will require much further investigation to be fully understood.

Fresh APL cell studies of retinoic acid resistance

A critical question is whether the in vitro findings related to ATRA-resistance in NB4 and HL-60 cells are relevant to clinical resistance in ATRA-treated APL patients. To date there has been but a single published report involving molecular analysis of the RARα region of PML-RARα/normal RARα in 20 APL patients, five of whom had relapsed from and were apparently resistant to ATRA therapy, and one of whom was reportedly primarily resistant to ATRA therapy [166]. Using a single-strand conformational polymorphism (SSCP) method, which typically has an estimated sensitivity to detect mutations of approximately 70%, no mutations were detected in the terminal three exons of PML-RARα, i.e., in the LBD of the RARα-region, in any APL specimen, in 29 specimens from other types of acute myelogenous leukemia, or in 25 specimens from various types of myelodysplastic syndrome [166]. Also, among 44 cell lines tested, including all major hematopoietic cell lines, positive results were only observed in two known HL-60 cell LBD mutants [62,63]. From these results, as well as SSCP analysis of all RARα exons included in PML-RARα, in seven APL cases, it was concluded that RARα

mutations are an infrequent event [166]. Recently, however, we have found missense mutations in the LBD of the RARα-region of PML-RARα in three ATRA-resistant APL patients from a select group of 11 APL patients who had relapsed from combined ATRA-chemotherapy treatment on an intergroup protocol (INT 0129) [2] and whose relapse APL cells had diminished in vitro sensitivity to ATRA-induced differentiation [167]. No mutations were found in the matched, ATRA-sensitive pretreatment specimens, indicating that the mutations were either acquired during protocol therapy or selected from rare mutant pretreatment APL cells. No mutations were found in the PML region of PML-RARα/PML of this same patient cohort [95]. Also, no mutations were detected in either the PML or RARα regions in seven protocol patients who either failed to achieve remission or who relapsed after receiving only chemotherapy. These results suggest that PML-RARα mutations account for a significant fraction of ATRA-resistant APL patients who relapse from combined ATRA-chemotherapy regimens, and they enhance the possibility that the molecular mechanisms defined in vitro culture systems will have clinical relevance. It is unknown if combination with chemotherapy had any influence on the incidence of PML-RARα mutations in APL patients receiving concurrent or sequential ATRA therapy. This would be of considerable interest to know, since relatively high-dose anthracyclines appear to yield superior clinical outcome in APL following chemotherapy alone [168], but could also lead to higher mutations rates in ATRA response molecules in combined-therapy regimens. Potential interactive effect of retinoids and chemotherapeutic agents, thus, could have important net effects related to clinical outcome.

P-glycoprotein and bcl-2

Two other molecular resistance mechanisms that merit particular mention are multidrug resistance (P-glycoprotein) and the bcl-2 gene family. In a study of ATRA-resistant HL-60 cells, we found no evidence for significant P-glycoprotein expression or cross-resistance to a variety of chemotherapeutic agents, despite the consideration that ATRA is an amphipathic agent like many multidrug-resistance-provoking compounds ([157]; R. Gallagher, V. Ling, and J. Gerlach, unpublished). However, another group has, more recently, found evidence for such cross-resistance in ATRA-resistant HL-60 cells [169,170]. We are not aware of any such studies in NB4 cells or of any specific studies in fresh APL cells after retinoic acid alone or in combination with chemotherapy. Related, inferential clinical data seem inconclusive, since, except at high levels of clinical resistance to chemotherapeutic agents that might involve many elements related to tumor cell progression, there is little evidence for cross-resistance to the chemotherapy- or ATRA-induced complete remission rates, although there could be significant effects on longer-term clinical outcome [74,108,141,171,172]. Clearly, further investigations are required in this area, particularly since the unusually favorable response of APL to chemotherapy alone or in combination with retinoids may be partly related to very low

constitutive levels of P-glycoprotein expression in this subtype of acute myelogenous leukemia [173].

The expression of the bcl-2 gene family, particularly high expression levels of bcl-2 itself or bcl-X_L, has also been related to the development of resistance to chemotherapeutic agents in myeloid leukemia cells [174–176]. Thus, as discussed above, the downregulation of bcl-2 mRNA and protein expression levels by retinoids could contribute to the enhanced clinical effect of chemotherapeutic agents in combination with retinoids, and the finding that ATRA-induced downregulation of bcl-2 is lost in the NB4-R1 subline [120] implies that ATRA-resistance might diminish its interactive action with chemotherapeutic agents. Further investigations are required to determine if variations in bcl-2 or other components of the apoptotic machinery could be primary factors in retinoid resistance. It will also be of interest to determine if arsenic trioxide, which also downregulates bcl-2 mRNA and protein levels in association with apoptosis in ATRA-sensitive NB4 cells [177], can modulate bcl-2 in ATRA-resistant APL cells, since such cells remain sensitive to arsenic-induced apoptosis in vitro and in vivo [178–180]. These results imply that retinoids and arsenicals have different regulatory effects on the bcl-2-associated programmed cell death pathway.

Alternative RARα fusion proteins

Overview

In addition to the PML gene, three other fusion gene partners due to alternative reciprocal chromosome translocations have been discovered in association with APL: PLZF, t(11;17)(q23;q21) [40]; NPM (nucleophosmin), t(5;17)(q32-35;q21) [181]; and NuMA (nuclear mitotic apparatus), t(11;17)(q13;q21) [182] (Table 1 and Figure 4). In all cases, a 5′ segment of each of these genes is fused to the same segment of RARα as in PML-RARα, i.e., to the coding exons for the B to F regions of RARα (Figure 4). This finding emphasizes the paramount importance of the RARα gene in the pathogenesis of APL and raises the possibility that the upstream fusion partner's primary role could simply be to provide a stable amino-terminal anchor for the formation of fusion gene-RARα protein dimers. However, as is the case for PML, there are indications that the fusion gene is not entirely passive. One indication of this is that careful cytologic studies of each variant describe subtle phenotypic differences from either the hypergranular or microgranular forms of PML-RARα-positive APL [41,113,183]. Another is that there is significant variation in sensitivity of the APL cells from the different variants to ATRA-induced differentiation: PLZF-RARα cells have little or no sensitivity either in vitro or in vivo [41,114], while leukemic cells from the other two variants do appear to have such sensitivity [112,113].

PLZF

PLZF is the second most common RARα fusion partner in APL, and much knowledge has been recently accumulated regarding the structure, expression, and possible function of the native molecule. In many respects, PLZF resembles PML, although much of the documentary evidence remains preliminary at this time. It is localized in punctate nuclear organelles [184–186], some of which appear to coincide with those containing PML [39]. Also, it appears to function as a cell-cycle-linked growth suppressor [39,187] and as a transcriptional repressor [39,188]. The latter activities are likely related to the amino-terminal POZ (POxvirus and Zinc finger) domain [186], which is a highly conserved protein–protein interaction motif associated with transcriptional repression [189]. PLZF markedly differs from PML, which is ubiquitously expressed, in its very limited range of tissue expression [40]. This range includes relatively high levels of expression in progenitor hematopoietic cells and lower levels of expression in committed myeloid cells, suggesting possible differential regulatory roles at different stages of hematomyelopoiesis [40,184]. Also, PLZF [40,184], but not PML [25,37], is downregulated in HL-60 and/or NB4 cells following ATRA exposure. Very recently, definitive evidence was provided for a direct interaction between the coiled-coil region of PML and a second zinc finger region near the carboxy terminus of PLZF that has a structure characteristic of a gene family homologous to the Drosophila zinc finger gene *Krüppel* [39]. This association may give an important clue to both the basis for the interaction between PML and PLZF and for the remarkable finding that these two genes are each associated as fusion products with RARα in APL. An important element of this clue is that other *Krüppel*-related proteins (called KRAB or *Krüppel*-Associated Box proteins) that function as transcriptional repressors have been found to interact with another class of nuclear proteins with a RING structure closely related to that of PML that serve as corepressors [190,191]. In this association, it is the KRAB proteins that bind to DNA, and, notably, the *Krüppel* region of PLZF has been determined to have specific DNA binding activity [188,192]. Thus, a not unreasonable speculation is that PLZF and PML may interact on a common set of gene targets through an interaction analogous to that between a KRAB protein and its RING protein corepressor, which, in turn, interact with other components of the transcription complex [190,191].

PLZF-RARα/RARα-PLZF structure and function

In the formation of the PLZF-RARα fusion gene in APL cells, the PLZF gene is broken after the second (usual) or third (one case) zinc finger of the nine-zinc-finger-long *Krüppel*-related domain [40,41]. The reciprocal RARα-PLZF fusion product incorporates 7 of 9 zinc fingers, including the five carboxy-terminal zinc fingers that have been implicated in specific DNA binding

[188,192] (Figure 4). Thus, this reciprocal fusion gene contains quite significant coding information and could be more important in the molecular biology of PLZF-RARα-associated APL than RARα-PML appears to be in PML-RARα-associated APL [40,185,192]. Additional heterogeneity is provided by alternative splicing of a PLZF exon that encodes a proline-rich segment and the presence of both RARα1-PLZF and RARα2-PLZF mRNAs, which are associated with different gene promoters.

Like PML-RARα, PLZF-RARα displays a mciroparticulate pattern in the nucleus of transfected cells [185,186] and, apparently, in t(11;17)-positive APL cells [39]. Following cotransfection, PLZF-RARα and PML-RARα colocalized to the same microparticulate structures, although it was not clear if this was due to the formation of PML-RARα:PLZF-RARα heterodimers [39]. However, unlike PML-RARα, PLZF-RARα did not displace PML from nuclear bodies onto the microparticulate structures. Since the reciprocal did occur, that is, PML-RARα did displace PLZF from nuclear bodies, this finding suggested that the lack of PML displacement by PLZF-RARα could be related to its content of only 2 of the 9 interacting *Krüppel*-like zinc fingers [39]. Following ATRA exposure, PLZF-RARα microparticulate staining was diminished and the protein was decreased on Western blot analysis, which suggested that, as for PML-RARα, ATRA might induce PLZF-RARα degradation [39]. These as yet unconfirmed and incompletely developed observations support the consideration that PLZF-RARα and PML-RARα may act on a convergent transcriptional regulation pathway but with different consequences.

Functional analyses of PLZF-RARα have demonstrated somewhat variable results. PLZF-RARα homodimers could bind to RAREs in the absence of RXR [185], and, thus, like PML-RARα homodimers [22], they have the potential of interacting with and modulating different promoters than normal RAR:RXR heterodimers. The RARE binding specificity of PLZF-RARα homodimers, however, differed from that of PML-RARα homodimers [186]. In the presence of RXR, PLZF-RARα RARE binding specificity paralleled that of RAR:RXRs but was much weaker [185]. Although the strength of binding could be increased by ATRA or by increased amounts of RXR, this strengthened binding led to the formation of multimeric complexes that could interfere with functional activity [185,186]. In transient transfection–reporter vector assays, PLZF-RARα was shown to inhibit endogenous ligand-dependent transactivation of the natural RARE for RARβ2 and of its trimeric form, in three different cell lines, including the myeloid leukemia cell lines HL-60 and U937 [193]. Using mutant PLZF-RARα constructs, the inhibitory activity was localized to the POZ domain complemented by the two *Krüppel*-like fingers [186]. Surprisingly, consistent stimulatory, rather than inhibitory, activity was observed in a separate report using the same PLZF-RARα and reporter plasmids, apparently related to the use of a different cell line and/or to technical differences [185]. In experiments in which PLZF-RARα was cotransfected with normal RARα plus the reporter vector, all studies showed

inhibition compared to the high transactivational activity of RARα alone [185,193]. In direct transfection experiments with U937 cells, another investigative group found no effect of transfected PLZF-RARα on the strong upregulation of RARβ2 mRNA by ATRA [122]. This difference from the reporter vector experiments might be related to differences between the environment of the native and recombinant RAREs. More generally, the latter investigators found that PLZF-RARα transfection of either U937 cells or RA-resistant HL-60 cells inhibited the differentiation response of these cells, as measured by several differentiation markers, compared to the effect of endogenous RARs or of transfected RARα or PML-RARα [122]. Both PLZF-RARα and PML-RARα inhibited the differentiative response of these myeloid leukemia cells to nonretinoid inducers [122]. Thus, while some experimental variability has been observed, the consensus is that PLZF-RARα has a more consistent dominant negative inhibitory effect on retinoid-responsive genes than PML-RARα and that this predominant negative activity is to a large extent dependent on the POZ domain, which is in keeping with the activity of related genes in other systems.

NPM-RARα

NPM is a well-recognized nucleolar phosphoprotein thought to have a role in ribosomal RNA processing or transport and has been linked to cell processes involving changes in cell proliferation [194]. It has also been identified as a fusion partner to alternative genes in some cases of two other malignancies, namely, anaplastic large cell lymphoma [195] and myelodysplastic syndrome [196]. Notably, the breakpoints of NPM are similar in all three associated diseases. The amino terminus of the NPM gene contains a putative metal binding site and a phosphorylation site (Figure 4), which is absent in one of the two alternatively spliced NPM-RARα transcripts expressed in the associated APL cells [181]. Messenger RNA for the reciprocal RARα-NPM was also detected in a cDNA library constructed from bone marrow cells from the propositus case. In transfection studies in CV-1 cells, both the long and short forms of NPM-RARα were shown to *trans*-activate two RAREs to a similar extent as normal RARα [181].

NuMA-RARα

In one case of atypical pediatric APL [113], RARα was found to be fused to a gene involved in nuclear mitotic assembly (NuMA). Structurally, the NuMA protein contains globular domains at both the amino and carboxy termini that are involved in nuclear reformation after mitosis, a long, central oligomerization domain likely involved in protein interactions, and a spindle-association domain towards the carboxy terminus [197,198]. In the formation of NuMA-RARα, the break in NuMA occurs near the carboxy terminus (Figure 4), excluding only the terminal nuclear reformation domain and nuclear localizing

sequences [182]. Although normal NuMA is alternatively spliced, only the major species was found in the fusion product, and the reciprocal RARα-NuMA mRNA was not detectable [182]. Notably, neither NuMA nor NuMA-RARα was associated with NBs, and there was no perturbation of PML distribution. There was, however, 'abnormal sheetlike nuclear aggregation' of NuMA-RARα, a pattern that was distinguishable from that of both normal NuMA and RARα, strengthening the concept that disruption of RARα-mediated function is of primary importance in APL [182].

The role of molecular diagnostics in the diagnosis and management of APL

Molecular methods: overview

Molecular techniques in current use in the diagnosis or monitoring of APL include Southern (DNA) and/or Northern (RNA) blotting, FISH, and RT-PCR. The exquisite specificity and, in some situations, sensitivity of these techniques offers tremendous potential to improve our ability to both diagnose and treat APL. However, the clinical utility, and hence ultimate usefulness, of such techniques needs to be validated in the context of prospective clinical trials; it is only in this setting that standardized methodology and rigorous statistical analysis can be combined to define the contribution of these assays to improvement of patient outcome. Although the current discussion will center on the role of RT-PCR in the diagnosis and management of APL, both FISH and Southern blotting can contribute complementary information that may be critically important, particularly in the analysis of cytogenetically atypical cases.

RT-PCR in the diagnosis of APL

The exquisite sensitivity and specificity of RT-PCR, coupled with its rapidity and prospects for quantitation and semiautomation, have led to its widespread use in both the diagnosis and monitoring of APL. In general, given the relatively high false-negative rate of cytogenetics and the sometimes difficult morphologic distinction between hypogranular APL and other AML subtypes, RT-PCR should be considered the diagnostic tool of choice in problematic cases where APL is a potential diagnosis. This conclusion is particularly true in the ATRA era, where the presence of the PML-RARα transcript predicts response to ATRA. As an example, in the recently completed U.S. and Canadian intergroup APL trial [2], 9 out of 230 (4%) molecularly evaluable cases (all of which passed central morphologic review) were PML-RARα negative; the cytogenetic findings in these cases were heterogeneous (none were 15;17-positive) but, more importantly, none of the five cases treated with ATRA alone achieved complete remission (CR) [65]. Finally,

performing the RT-PCR assay at diagnosis of APL allows determination of the PML breakpoint site (or PML-RARα mRNA type); although PML-RARα isoform type (S, L, or V) has not been proven to be of prognostic value [65,74], its determination facilitates subsequent MRD evaluation by defining the most appropriate primer set to use in follow-up testing.

RT-PCR for detection of MRD

It is in the area of MRD detection that RT-PCR is likely to find its major role, but there are a number of complex technical and clinical issues that remain to be resolved. At present, there is a complete lack of standardization among laboratories that perform the PML-RARα and RARα-PML RT-PCR assays; this lack of standardization precludes extrapolation of results from a single laboratory to a larger clinical setting. Furthermore, despite claims to the contrary (see below), there is as yet no definitive study that has unequivocally proven the prognostic value of MRD detection in APL. It is imperative, and would seem self-evident, that patients not be subjected to intensive therapeutic regimens based on the results of an assay that has not yet been standardized. With these caveats, it is nevertheless useful to review the current state-of-the-art of MRD detection in APL using RT-PCR for detection of PML-RARα and/or RARα-PML transcripts.

Early studies

In 1994, Diverio et al. [199] published a summary of early data from different laboratories regarding the predictive value of a positive or negative PML-RARα assay at completion of consolidation therapy for APL. In that summary [199], 36 of 122 heterogeneously treated patients were PML-RARα positive (RT-positive) after consolidation, while 86 were negative (RT-negative). Among the 36 RT-positive patients, 27 subsequently relapsed within six months of the last positive RT-PCR assay, whereas only 5 of the 86 RT-negative patients relapsed ($p < 0.001$). Although compelling, these data were generated from a heterogeneous group of newly diagnosed or relapsed patients, some of whom received ATRA alone for treatment of their APL. Furthermore, no attempt was made to control for different sensitivity levels of the RT-PCR assay in different laboratories. Thus, although provocative, these results should be regarded as preliminary and reflective primarily of insensitive assays that, not surprisingly, identify patients with large leukemic burdens.

Technical improvements in the RT-PCR assay

Several recent reports have been published outlining potential improvements in the technical aspects of the PML-RARα assay [200,201]. Thorough denaturation of RNA prior to reverse transcription, as well as thorough initial denaturation of first-round PCR products, results in a routine sensitivity level of at

least 1 in 10^5 or, more commonly, 1 in 10^6 ([201,202] and our unpublished data). Inefficiency of the reverse transcriptase step seems to be responsible for the previously described poor RT-PCR sensitivity in the PML-RARα assay [201]. We and others [200] have also found hot-start PCR to be useful in decreasing the incidence of false PCR products. Most groups are now using a version of this 'new' assay, which renders interpretation of previously published results problematic.

Recent studies

More recent studies [203–206] tend to support the notion that a positive RT-PCR assay, at some point after consolidation therapy, predicts eventual relapse. However, the sensitivity level and timing of the assay remain critical issues that must be addressed systematically. In addition, the predictive value of a negative RT-PCR result is at present unclear. For example, Grimwade et al. [207] studied 12 patients with de novo APL who achieved CR but subsequently relapsed despite induction therapy with ATRA and aggressive consolidation chemotherapy. Nine of these 12 relapsed patients were PCR negative after one or more courses of consolidation therapy, suggesting that a negative RT-PCR assay, at least at the sensitivity level and time points studied by Grimwade et al. [207], has marginal predictive value. Using a version of the newer, sensitive RT-PCR assay, we analyzed nine L-form APL patients who had been in sustained clinical CR for at least one year. Two of these cases were PML-RARα positive, while four cases, including three cases not detected by the PML-RARα assay, were RARα-PML positive (see below). In a preliminary report of the Italian AIDA trial [208,209], it was reported that 96% of patients were RT-PCR negative at the end of consolidation; it should be noted that these investigators used a relatively insensitive RT-PCR assay (sensitivity 1 in 10^4). In that study [208], patients who were PCR positive at the end of induction therapy (59 of 139 patients, or 39.5%) had an equivalent outcome to those who were PCR negative. Further work is clearly needed to establish the incidence of PML-RARα and/or RARα-PML positivity, using more sensitive assays, in APL patients at various times after therapy and in long-term CR.

Detection of RARα-PML

The RARα-PML transcript, derived from the der17 chromosome, can be detected in 67%–80% of newly diagnosed APL cases [104–106,210]. Although the presence or absence of RARα-PML per se does not appear to influence prognosis in APL [106], it provides an alternative, perhaps more sensitive, assay for MRD detection. For example, several groups [210,211] have reported that the RT-PCR assay for detecting RARα-PML is at least one order of magnitude more sensitive than a similarly performed assay for detection of PML-RARα. In one study [211], bone marrow samples from 18 APL patients in long-term remission were analyzed using nested RT-PCR assays for both

PML-RARα and RARα-PML. The PML-RARα assay (sensitivity level: one cell in 5×10^4) was negative in 18 of 18 patients, while the RARα-PML assay (sensitivity level: one cell in 5×10^5) was positive in 6 of 18 long-term CR patients, two of whom had undergone allogeneic bone marrow transplantation. These results are reminiscent of the situation in AML patients with t(8;21), in whom molecular evidence of the 8;21 translocation is commonly found in long-term CR, including patients post-allogeneic transplant [212,213]. The clinical relevance of such results is at present unclear; however, at a minimum they require confirmation in carefully controlled, prospective studies using standardized treatment regimens.

Unresolved questions and issues for further study

Molecular techniques, particularly RT-PCR, can serve as valuable adjuncts in the diagnosis of APL, particularly in cases where morphology or cytogenetics are either atypical or provide disparate information. The most important recent finding is the presence of either PML-RARα or RARα-PML transcripts in APL patients in long-term CR. Thus, it cannot now be stated that a positive RT-PCR assay is predictive of certain relapse in APL. The development of standardized, quantitative RT-PCR techniques will hopefully address the issue of interlaboratory variation and allow an answer to the basic question of MRD biology: that is, at what time point, and at what sensitivity level, should the RT-PCR assay be performed to provide the most clinically relevant information? Recent studies in other hematologic malignancies suggest that there is a level of MRD at which host immunologic mechanisms can provide tumor control [214]. Defining this level in APL represents the next and perhaps most difficult challenge for investigators in this field.

Summary

The preceding two years have witnessed an explosion in the accumulation of knowledge relating to the molecular pathogenesis of APL. Critical advances include:

- The molecular delineation of atypical APL cases with alternative RARα fusion partners, and the demonstration that cells from 2 of the 3 types of 'atypical' APL retain sensitivity to ATRA. Perhaps the key question is why such cases are so rare. However, at a minimum, the presence of such cases argues persuasively that disruption of the retinoid signaling pathway is a (perhaps *the*) key pathogenetic feature of APL. Although certainly not 'passive' partners, it is likely that PML, PLZF, NPM, and NuMA serve similar functions in the pathogenesis of APL.
- The demonstration, in transgenic mice, that PML-RARα (and PLZF-RARα) can disrupt normal hematopoiesis and, given sufficient time, cause an APL-like syndrome. The variation in phenotype of the mice, which

appears to be a consequence of the specific expression vector used, emphasizes the cell-type-specific nature of PML-RARα function.

- Continuing functional analysis of PML, PLZF, and RARα. In particular, the demonstration that PML and PLZF can form heterodimers provides a critical functional link between these proteins and offers a tantalizing glimpse at how both, when linked with RARα, can cause APL.
- The demonstration that PML-RARα is degraded, perhaps via a ubiquitin-dependent pathway, in response to ATRA. This result offers a unifying, if not yet proven, hypothesis to explain the sensitivity of leukemic promyelocytes to ATRA. Unfortunately, it is not known if ATRA can also cause degradation of NPM-RARα or NuMA-RARα (atypical cytogenetic APL variants that retain ATRA responsiveness). Whether PML-RARα degradation is a cause, or consequence, of promyelocytic maturation remains unclear.
- Continuing insight into retinoid resistance, including the first demonstration of mutations in the PML-RARα molecule from ATRA-resistant patients.
- The definitive demonstration that the two major PML-RARα isoforms, while having subtle differences in biological activity and producing slightly different APL phenotypes, nevertheless do not, in and of themselves, have prognostic significance in patients treated with ATRA/chemotherapy combinations.
- Further functional analysis of PML-RARα in vitro. The fascinating finding that PML-RARα is cytotoxic to most cell types suggests that it must function as an oncogene in a very specialized milieu. In addition, the demonstration that both the DBD (from RARα) and dimerization interface (from PML) are required for full in vitro functional activity, coupled with the finding that PML itself is a strong transcriptional suppressor, suggests that PML-RARα may directly repress transcription of RA target genes.

The challenge in APL research now is to integrate the above findings into a cohesive, unifying model that explains the biology of APL at a molecular level. The creation and validation of such a model will clarity whether APL is a fortunate medical curiosity or whether it will serve as a paradigm for the development of effective differentiation therapies in other types of human cancers.

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5. Current approaches to acute promyelocytic leukemia

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Clinical characteristics of acute promyelocytic leukemia

Acute promyelocytic leukemia (APL), the M_3 subtype of acute myeloid leukemia (AML) in the French–American–British (FAB) classification system [1], is a distinct clinical and pathologic subtype that accounts for approximately 10% of acute myeloid leukemia (AML) cases. The unique features of APL have been well described [2–4] and include a characteristic morphologic appearance [5], a reciprocal translocation between the long arms of chromosomes 15 and 17 [6], younger age of onset [7], and severe consumptive coagulopathy with a high incidence of early fatal hemorrhage [3,8–10]. This coagulopathy is frequently exacerbated by cytotoxic chemotherapy, probably a result of leukemia cell lysis with release of procoagulant intracellular contents [11,12].

Diagnostic criteria for APL

Because the therapy for APL must now differ from other subtypes of AML, the critical initial act is to make an accurate diagnosis. The availability of therapeutic agents that are uniquely suited to treat APL necessitates quick and accurate diagnosis. Light microscopic evaluation of bone marrow aspirate smears provides the initial hint of the correct diagnosis. The classical FAB M_3 morphology includes hypergranular cells and multiple Auer rods. Variant forms may present with hypogranular bilobed cells, basophilic cells, differentiated promyelocytes, and a few blast cells (M_2-like), with another form consisting largely of blast cells and a few early promyelocytes (M_1-like) [13,14].

Immunophenotype provides supportive evidence of the diagnosis of APL. A pattern of CD33 and/or CD13 positivity and CD14 and HLA-DR negativity is seen in 96% of cases. CD2 was positive in the FAB variant and in the subtype with basophilic cells, but negative with other subtypes [14]. The clinical correlates of molecular and immunophenotypic subtypes, including expression of CD34, CD2, and CD19, remain controversial [15].

The detection of chromosomal translocation t(15;17) provides conclusive

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evidence of the diagnosis of APL. In addition to standard metaphase evaluation of karyotype, fluorescence in-situ hybridization probes may also be used to make a cytogenetic diagnosis [16–19].

Since variant translocations are also found, further evaluation by RT-PCR for the PML/RARA transcript is valuable in confirming the diagnosis and providing a tool to evaluate patients throughout their therapy for residual disease [20–24]. The discovery of the ability of retinoic acid to induce clinical remissions was paralleled by the description and explication of the molecular genetic basis of the disease. The molecular biology of this disease is detailed in the previous chapter, and only the applications of these data will be discussed in this chapter. The availability of rapid highly specific and sensitive RT-PCR assay using microfuge extraction of RNA and a single amplification round may speed the diagnosis of APL [25].

An alternative to molecular testing is the use of monoclonal antibodies directed against PML in an immunofluorescent assay [26,27]. This antibody can demonstrate the disruption of the PML nuclear bodies to produce a change of the nuclear staining pattern from speckled (wild-type PML protein) to microgranular (PML-RARα fusion protein). Because the epitope identified by PG-M_3 is located in the amino terminal portion of PML, it can detect APL cases characterized by breakpoint occurring at different sites of PML (bcr-1, bcr-2 and bcr-3) [28]. This method provides a rapid, sensitive, and highly specific test for the diagnosis of APL that bears the t(15;17).

Therapy of APL

The therapy of APL was transformed by the discovery that all-retinoic acid can induce terminal differentiation and apoptosis of the malignant leukemic cells, resulting in a clinical remission [29–33]. This observation provided the first evidence of effective differentiation therapy and also provided a rationale for biologically targeted therapy based upon subtype of leukemia.

While combinations of anthracyclines and cytosine arabinoside were able to induce clinical remissions of long duration in patients with APL at a higher frequency that other FAB subtypes, historical and randomized comparisons of regimens that included all-*trans* retinoic acid (ATRA) have shown substantial superiority. Therefore, a retinoid should be included as a standard component of antileukemic therapy for patients with APL. The optimal dose, duration, and sequence of retinoid-containing therapy in APL remains uncertain.

Role of ATRA therapy in APL

The concordance of studies in APL therapy with ATRA suggests that there is a benefit to addition of ATRA to induction therapy. The question of whether ATRA induction without chemotherapy offers benefits compared to ATRA

induction with chemotherapy has not been answered by randomized trials. However, the achievement of complete remission (CR) rates of 90% with the combination approach suggests that there may be no need to perform such a study. The additional benefit of early application of chemotherapy might be to prevent the morbidity and mortality of the retinoic acid syndrome. Furthermore, there may be a synergy between retinoids and cytotoxic therapy [34].

Pharmacology of ATRA

ATRA and the other retinoids are important regulators of cellular proliferation and differentiation [35,36]. The retinoids are derived from retinol or vitamin A; ATRA is a natural metabolite of retinol, with a normal plasma concentration of 1.5–3.0 ng/mL [37,38], with similar levels in patients with APL and normal volunteers [38].

ATRA administered as a single oral dose of 45 mg/m^2 achieves peak plasma concentration approximately 1–2 hours after ingestion [38,39]. Median peak plasma concentrations of 300–400 ng/mL have been reported but with great interpatient variability (ranges of 30–2500 ng/mL) [40,41]; these concentrations are within the range that induces in vitro differentiation of leukemia cells. ATRA is rapidly eliminated from plasma, with a median half-life of approximately 45 minutes (range 17–77 minutes). Plasma levels return to the physiologic range in 6–12 hours [38,39]. ATRA is extensively bound (>95%) to plasma proteins, mainly to albumin. Metabolites in plasma and urine include 13-*cis* retinoic acid, 4-oxo-*trans* retinoic acid, 4-oxo-*cis* retinoic acid, and 4-oxo-*trans* retinoic acid glucuronide [38,39]. ATRA does not appear to penetrate into cerebrospinal fluid [38]. ATRA absorption is improved with food and should be taken immediately after meals to improve bioavailability [38,39,42]. Single doses of oral ATRA at 15 mg/m^2 provide a maximal plasma concentration (C_{pmax}) high enough (10^{-6} M) to induce APL cell differentiation [43].

A wide range of doses from 10 to 100 mg/m^2 have been used with no clear dose–response effect, but rigorous testing has not been performed. The most common dosage in clinical trials has been 45 mg/m^2 daily as a single dose or in two equally divided doses. Because the initial Chinese studies used a dose of 45 mg/m^2, this dose has been adopted as the one most frequently used because it was effective. Doses of 25 mg/m^2 were effective, but did not reduce the incidence of retinoic acid syndrome [44]. ATRA at the dose of 15–20 mg/m^2/day in 26 de novo APL patients allowed 92% of patients to achieve clinical CR after 13 to 67 days of ATRA treatment. Of note, none of these patients experienced retinoic acid syndrome (RAS) or disseminated intravascular coagulopathy (DIC) [43]. Patients entered remission with as few as 13 days of ATRA therapy [43]. The optimal or necessary dose or duration of ATRA is unknown.

Chronic oral ATRA therapy in patients with APL results in progressively lower peak plasma concentrations and area under the concentration–time curve [38,43,45]. These decreases occur early, with a decrease of more than 50% in the mean peak plasma concentration by day 7 of therapy [38]. Doubling the ATRA dose has not yielded increases in the plasma concentration [45]. This decrease in plasma concentration is associated with a nearly 10-fold increase in urinary excretion of 4-oxo retinoic acid glucuronide. These data suggest that increased metabolism, possibly by a cytochrome P-450-like enzyme, may account for the increased plasma clearance [38]. Other postulated causes of the decline in plasma levels include an increase in retinoic acid binding proteins [46,47] and decreased absorption of oral ATRA. These data suggest that resistance to ATRA may result in part from exposure to inadequate concentrations of ATRA [48,49], but an in vitro decrease in sensitivity to the differentiating effects of ATRA on human APL cells at relapse has also been demonstrated [47]. Intermittent dosing of retinoids may preserve their therapeutic effect [49,50]. Administration of ATRA on a continuous daily schedule results in a rapid and sustained decrease in plasma drug concentrations, whereas plasma ATRA concentrations on an every-other-week schedule evaluated up to 11 weeks were equivalent to baseline. This finding suggests that an intermittent schedule of ATRA administration may allow for ongoing effective therapy [51].

ATRA can be mixed with difficulty for nasogastric administration in patients who are intubated [52]. The contents of the capsules can be mixed in 20 mL of sterile water and then heated to 37° Celsius. Liposomal tretinoin is undergoing investigation and may be available on a compassionate basis for intravenous administration in intubated patients.

Other agents, including neplanocin A, have been shown to potentiate the effects of ATRA on APL cell lines [53]. Vitamin K_2 may enhance ATRA effects and induce apoptosis [54]. Similar synergistic activity has been reported with vitamin D analogues [55].

Clinical trials of ATRA

In vitro studies had demonstrated induction of differentiation of promyelocytic leukemia cells [56,57], and case reports had described clinical responses to 13-*cis* retinoic acid in patients with APL [58,59]. The first patients were treated with ATRA in Shanghai [60]. The initial results were dramatic [61]. Exploratory phase II trials in China, France, and New York established the efficacy of ATRA in APL. As reviewed elsewhere [2,62,63], subsequent clinical trials have confirmed the activity of ATRA in patients with APL, with complete remission rates of approximately 80% [63–77]. Response rates in patients previously treated with chemotherapy, but not ATRA, have been similar to those in patients with untreated APL. These data support the use of ATRA alone or in combination with an anthracycline-containing chemotherapy regimen in patients with untreated APL [63,71–73,78–85]. ATRA is

indicated for induction of remission in patients with APL (FAB-M3) with the characteristic translocation between chromosomes 15 and 17 and/or the PML/RARα gene.

In contrast to conventional chemotherapy drugs, ATRA initially induces cellular differentiation in APL cells rather than cell lysis. This mechanism of action would be expected to decrease the initial mortality associated with hemorrhagic complications. Results of early clinical trials have confirmed the value of ATRA in acute promyelocytic leukemia. However, the major impact of ATRA in randomized trials has been to improve leukemia-free and overall survival, with little effect on early mortality.

Despite the impressive remission rates achieved with ATRA, remission duration following treatment with ATRA alone has been uniformly short; Frankel et al. reported a median remission duration of 3.5 months (range 1–23 months) in 13 patients on continuous maintenance ATRA; only 3 of 19 patients who relapsed after ATRA therapy achieved a second remission with this drug [71]. These data have stimulated clinical trials combining ATRA with conventional chemotherapy using either a sequential or concomitant schedule. Alternative approaches to induction therapy include the use of retinoids alone or combination of retinoid and anthracycline therapy, with and without the use of cytosine arabinoside.

Long-term follow-up is now available from studies performed in New York, Europe, and Japan. In the New York trial of 65 newly diagnosed and 30 previously treated retinoid-naive patients with molecularly confirmed diagnoses of APL, 83% of patients achieved a CR [71,76,78]. All induction failures in molecularly diagnosed cases were due either to early death or to premature withdrawal. Cytotoxic chemotherapy was not routinely introduced in these patients or for elevated leukocyte counts. These patients received either three cycles of idarubicin and cytarabine-based therapy or monoclonal antibody therapy [86]. Median disease-free and overall survival rates for all newly diagnosed patients are more than 40+ and more than 43+ months, respectively. In a subset of 27 newly diagnosed patients treated in 1990–1991 whose actual median follow-up period is now more than five years, median disease-free and overall survival rates are greater than 57+ and greater than 58+ months, respectively, with 56% of these patients alive in first remission. This outcome compares favorably to a historical control group of 80 patients treated with chemotherapy alone whose median disease-free and overall survival rates were 11 and 19 months, respectively, with only 22% of these patients alive in first remission at five years [76]. Although a high proportion of previously treated patients also achieved a CR after RA treatment, median disease-free and overall survival rates noted for that group were markedly lower (i.e., 7.5 and 10.9 months, respectively) [76].

The Japan Adult Leukemia Study Group (JALSG) combined ATRA with chemotherapy and compared to their prior results with chemotherapy without ATRA [74,75]. Patients were scheduled to receive 45 mg/m^2 oral ATRA daily until achievement of a CR. If patients had initial leukocyte counts of more

than 3.0 × 10(9)/L, they received 40 mg/m² daunorubicin (DNR) for thre days and 200 mg/m² behenoyl cytarabine (BHAC) for five days in addition t ATRA. The chemotherapy was also added at any time during remissio induction therapy if the patients showed peripheral blood myeloblast an promyelocyte counts of more than 1.0 × 10(9)/L. Three courses of consolida tion and six courses of maintenance/intensification chemotherapy were admin istered. Of 196 evaluable patients, 173 (88%) achieved a CR: 59 of 62 (95% treated with ATRA alone, 41 of 49 (84%) treated with ATRA plus late chemotherapy, 63 of 73 (86%) treated with ATRA plus initial chemotherapy and 10 of 12 (83%) treated with ATRA plus both initial and later chemo therapy. The CR, early relapse, and mortality rates were improved with th use of ATRA and chemotherapy. At a median follow-up of 36 months, th predicted four-year event-free survival (EFS) rate for 196 evaluable patient and the four-year disease-free survival (DFS) rate for the CR cases were 54% and 62%, respectively. Predicted four-year overall survival was 74%. Th EFS was significantly improved compared to the prior two chemotherap approaches of this group, and there was a trend to improved overall surviva [74,84].

Two randomized trials have now been completed to compare ATRA t conventional chemotherapy for induction of remission in patients with un treated APL. The European APL 91 study randomized patients to receiv ATRA or chemotherapy (CT) with cytosine arabinoside (ara-C) plu daunorubicin. Patients randomized to initial treatment with ATRA receive the same chemotherapy when they achieved a complete remission or earlie for specific threshold levels of leukocytosis; approximately one third of pa tients in the ATRA group received chemotherapy with ATRA initially fo white blood cell counts of 5000/mL or more, and another third had chemo therapy added for a rising white blood cell (WBC) count. All patients wh entered remission received two consolidation courses. The first consolidatio was daunorubicin 60 mg/m² for three days and cytarabine 200 mg/m²/day fo seven days. The second consolidation used a daunorubicin dose of 45 mg/m for three days and cytarabine 1 g/m² every 12 hours for eight doses. This tria was terminated early when the ATRA arm was found to have a superior EF [87]. The complete remission rate was 91% in the ATRA group and 81% i the CT group ($p = 0.25$). Early mortality and incidence of resistant leukemi were also not significantly different for the two arms. Updated analyses of thi study confirm the early results, with an estimated EFS at one year of 83% i the ATRA group compared to 50% in the chemotherapy group ($p = 0.0001$ [63,72,73,88]. Similarly, the estimated risk of relapse at one year was 13% versus 41% ($p = 0.0006$), and the survival at one year was 91% versus 74% ($p = 0.01$) in favor of the ATRA arm. With more than four years of follow-up estimates of relapse were 31% at four years in the ATRA group versus 72% in the CT group ($p = 0.0001$); predicted EFS was 62% at four years in th ATRA group versus 15% in the CT group ($p = 0.0001$); and survival was 76%

at four years in the ATRA group versus 49% in the CT group ($p = 0.005$) [88]. In addition, coagulopathy was reversed more rapidly in the ATRA arm (mean: two days) versus the chemotherapy arm (mean: five days).

The European APL Group is now randomizing patients to receive sequential ATRA → chemotherapy as described above or concomitant ATRA plus chemotherapy using the same drugs (APL93) [63,72,73,89]. In this trial, therapy was stratified by age group and initial leukocyte count. Patients with initial WBC less than 5000/mm^3 were randomized between ATRA (45 mg/m^2/day until CR) followed by CT, as in the APL 91 trial (ATRA → CT group) or ATRA with CT started on day 3 (ATRA + CT group). Patients with WBC greater than 5000/mm^3 received ATRA and CT from day 1 (high WBC group). Patients over age 65 received ATRA → CT (elderly group). All patients who achieved a CR received the same two courses of anthracycline and AraC CT consolidation as in the APL 91 trial. After completion of consolidation therapy, patients underwent a second randomization between one of four maintenance arms: 1) ATRA 45 mg/m^2/day for 15 days every three months, 2) continuous 6-mercaptopurine (6 MP 90 mg/m^2/d) + methotrexate (MTX 15 mg/m^2/week), 3) both ATRA and 6-MP/MTX, or 4) observation.

Interim analysis. Overall, 92% of patients achieved a remission, with 89% of the elderly patients entering remission. Patients in the elderly group had a higher incidence of early death (11%), as did patients with higher WBC (9%). ATRA syndrome occurred in 15% of patients but was fatal in less than 2% of the entire population. EFS at two years ranged from 80% in the group that received ATRA + CT to 66% in the elderly, with the highest relapse rate at two years of 29%, in the group with the elevated WBC. The elderly group ($p = 0.009$) and the high WBC group ($p = 0.004$) had significantly lower EFS than patients less than 65 years old with WBC less than 5000/mm^3. The high WBC group had more relapses at two years (28%) ($p = 0.003$) than patients with WBC less than 5000/mm^3. In the latter, more relapses were seen in the ATRA → CT than in the ATRA + CT group ($p = 0.008$), but no significant differences in EFS and survival between these two groups were observed. There is a trend toward patients maintained with ATRA to have a lower risk of relapse and a longer EFS. There was a significant doubling of the rate of relapse in patients who did not receive maintenance chemotherapy and a decrease in EFS. No interaction was found between ATRA and low-dose chemotherapy. Whether this benefit translates into better survival will have to be determined with longer follow-up [89].

The North American Intergroup 0129 study randomized 346 evaluable patients with untreated APL to receive oral ATRA 45 mg/m^2 daily or standard induction chemotherapy with ara-C plus daunorubicin. All patients who achieved complete remissions received one course of the same chemotherapy regimen used for the chemotherapy induction and a second high-dose cytarabine and daunorubicin consolidation. All patients in continuing remission were then randomized to observation or ATRA maintenance 45 mg/m^2

daily for one year. Therefore, patients who completed therapy fit into one of four groups. In contrast to the European study, only 5% of patients in the ATRA arm were crossed over to receive chemotherapy during induction. The complete remission rate was lower than the CR rates reported by most other large phase II or III trials in APL: 69% in the chemotherapy group and 72% among those given all-*trans*-retinoic acid ($p = 0.56$). This study failed to show a large decrease in treatment-related induction deaths: 13.5% of patients who received chemotherapy compared to 11% in the ATRA arm ($p = 0.50$). However, among patients who achieved remission, the estimated one-year disease-free survival (DFS) was 92% in the ATRA arm versus 57% for the chemotherapy arm ($p < 0.0001$). A similar advantage was seen in estimated one-year DFS for patients randomized to maintenance ATRA (77%) versus observation (56%) ($p < 0.0001$). When the combined effects of induction and maintenance were examined the relapse rate and estimated one-year DFS for patients who never received ATRA (i.e., chemotherapy induction and no maintenance after completion of consolidation) were significantly inferior compared to the other three groups [83]. When both induction and maintenance treatments were taken into account, the estimated rates of DFS at one, two, and three years were, respectively, 77%, 61%, and 55%, for patients assigned to chemotherapy and then ATRA; 86%, 75%, and 75% for ATRA and then ATRA; 75%, 60%, and 60% for ATRA and then observation; and 29%, 18%, and 18% for chemotherapy and then observation. By intention-to-treat analysis, the rates of overall survival at one, two, and three years after entry into the study were 75%, 57%, and 50%, respectively, among patients assigned to chemotherapy, and 82%, 72%, and 67%, respectively, among those assigned to ATRA ($p = 0.003$). In this trial, ATRA did not improve the complete remission rate or decrease the induction death rate compared to conventional induction chemotherapy, but exposure to ATRA during either induction or maintenance significantly improves disease-free and overall survival as compared with chemotherapy alone.

Another approach has been to administer idarubicin with ATRA to all patients during induction. Estey and colleagues at M.D. Anderson Cancer Center treated patients with ATRA 45 mg/m^2 daily until CR and idarubicin 12 mg/m^2 daily for four days beginning on day 5 of ATRA. Patients in CR received two courses of idarubicin 12 mg/m^2 daily for three days and then, until two years post-CR date, alternated three cycles of mercaptopurine, vincristine, methotrexate, and prednisone (POMP) with one cycle of idarubicin 12 mg/m^2 daily for two days. They reported a CR rate of 77%, a DFS at one year of 87% in patients who achieved a CR, and 67% for the group as a whole. The outcome in the 43 patients treated in this fashion was superior to the prior cohort of patients, who were treated without ATRA. The authors suggest that omission of cytarabine from the treatment program might allow increased delivery of anthracyclines with a beneficial outcome [82,90].

The Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) in cooperation with the Associazione Italiana di Ematologia ed

Oncologia Pediatrica and some European Organization for Research and Treatment of Cancer (EORTC) centers followed up on a pilot trial combining ATRA administered orally beginning on the first day of induction at the dosage of 45 mg/m²/day until CR, with idarubicin intravenously at the dosage of 12 mg/m²/day on days 2, 4, 6, and 8 of the induction. Patients who achieved CR were consolidated with three courses of chemotherapy without ATRA; thereafter, they were followed up for molecular and hematologic CR [79–81]. The three chemotherapy consolidations included idarubicin 5 mg/m²/day on days 1–4 with cytarabine 1 g/m² IV over six hours on days 1–4; mitoxantrone 10 mg/m² IV and etoposide 100 mg/m² IV on days 1–5; idarubicin 12 mg/m² on day 1, cytarabine 150 mg sc q eight hours on days 1–5, and 6-thioguanine 70 mg/m² q eight hours on days 1–5. In this multicenter trial, AIDA LAP 0493, adult and pediatric APL patients with molecular or cytogenetic studies for PML-RARα hybrid gene or t(15;17), which confirm the diagnosis, are enrolled to receive the same induction and consolidation therapy of this pilot study. After consolidation, patients who are RT-PCR- for PML-RARα hybrid gene are randomized to four arms, whereas patients who are RT-PCR+ after consolidation undergo, if eligible, an allogeneic transplantation procedure [80].

The results of 240 patients enrolled in this trial have been reported. The median age was 38 years. CR was achieved in 95%, the remaining 5% dying of complications. There was no resistant disease. RT-PCR converted to negative in 60% of patients after induction and was negative in 98% of patients at the end of consolidation. After a median follow-up of 12 months, the estimated actuarial EFS was 79% at two years [79]. The exceptional early results of this trial suggest that this approach should be considered as standard therapy.

The British MRC ATRA Trial compared a short five-day preinduction course of ATRA versus an extended course starting at the same time as chemotherapy and continuing until remission was achieved (or a maximum of 60 days). The extended administration schedule was more effective, resulting in improved CR rate (89% vs. 67%), fewer early deaths (12% vs. 23%), less resistant disease (2% vs. 9%), and improved overall survival (74% vs. 53%) [91]. The subgroup of patients with elevated WBC of more than 10×10^9/L faired poorly, with no significant benefit of the extended use of ATRA. CR was 63% and three-year survival was 37% for the extended ATRA group, compared with 44% and 58%, respectively, in the short ATRA therapy cohort. The results in patients with WBC of less than 10×10^9/L are more typical of other large studies and showed a benefit of the extended schedule. CRs were 94% versus 76% ($p = 0.0001$) and three-year survival 85% versus 60% ($p = 0.001$) for extended versus short arms [91].

ATRA induction in an Argentinian series led to a CR rate of 78%, 14% fatal hemorrhage, and 4% fatal RA syndrome. DFS in responders was 81% at one year and 74% at two years [92].

Complete remissions achieved with ATRA have generally required approximately 5–6 weeks of treatment, similar to the time required to achieve

complete remission after conventional chemotherapy [2,62,65]. These remissions appear to occur more gradually and without intervening bone marrow aplasia. A major anticipated advantage of ATRA induction was reduction of hemorrhagic morbidity and mortality. While hemorrhagic morbidity has generally occurred less frequently, and pretreatment disseminated intravascular coagulation has resolved more rapidly with ATRA [63,72,73,87], mortality has not been significantly reduced in randomized trials. Management of patients with elevated WBC remains problematic. There has not been a benefit to leukapheresis. The addition of early chemotherapy improved the outcomes in the European series. Hydroxyurea has been used without documented success. Prophylactic addition of steroids may be useful for these patients [93].

Alternative approaches to induction therapy include the use of retinoids alone and the combination of retinoid and anthracycline therapy, with and without the use of cytosine arabinoside.

Postremission therapy

Once patients are in remission, the optimal postinduction therapy remains to be determined. Since remissions that are not consolidated with anthracycline-based chemotherapy are short-lived, some cytotoxic therapy should be administered. While the dose of anthracycline may be related to outcome, with increased doses having superior results, the role of cytarabine is more controversial. Data from the Southwest Oncology Group suggests that higher doses of cytarabine administered before the advent of ATRA led to inferior outcomes [94]. This finding is indirectly supported by the subset analysis of CALGB 8525, in which outcomes were inferior in the high-dose cytarabine consolidation cohort compared to intermediate dose of cytarabine [95,96]. Investigators at M.D. Anderson Cancer Center have argued that elimination of cytarabine would allow intensification of anthracycline dosing and might provide for a superior outcome [82]. However, recent reports of a German study suggests a value of high-dose cytarabine consolidation [97]. Prior to the use of ATRA during induction, there was a 67% nine-year DFS in the group consolidated with high-dose cytarabine-based therapy compared to 21% in the group who received reduced doses of cytrarabine. In subsequent studies incorporating ATRA, the CR rate and DFS appear to be promising. The European APL study has used a 1 g/m^2 dose of cytarabine in consolidation [89].

ATRA as postremission therapy following induction chemotherapy may prolong remission [98,99]. ATRA 45 mg/m^2/day for 10 weeks was administered to eight patients. After a median follow-up of 29 months, seven patients remain in complete remission; one patient relapsed at 26.9 months. Two patients with persistence of PML/RARα rearrangement detected by RT-PCR analysis after induction chemotherapy converted to negative following ATRA. Toxicity of ATRA given in the postremission setting was mild and consisted of headache, dry skin, and elevations of triglycerides and transami-

nases. No patient developed evidence of the retinoic acid syndrome. ATRA administered during induction therapy has typically been stopped prior to administration of cytotoxic chemotherapy consolidation therapy. However, there is no firm basis for this practice courses of ATRA could be continued during postremission chemotherapy with possible synergy.

The benefit of maintenance ATRA therapy or chemotherapy continues to be investigated. As mentioned above, repeated dosing of anthracyclines and/or the use of mercaptopurine and methotrexate-based maintenance pulses have been used. Only the European APL 93 study provides evidence of benefit of this additional therapy, with decreased relapses observed in the maintenance arms. Whether the maintenance therapy is effective in patients with high-risk features — age greater than 65 years or WBC greater than 5 — remains to be determined [89]. The North American Intergroup study suggests not only that should retinoic acid be included during induction therapy but also that postremission retinoid therapy may be helpful [83]. The earlier findings that continuous exposure to ATRA leads to drug resistance and relapse supports the hypothesis that intermittent dosing schedules may be a better approach for maintenance therapy. This approach continues to be studied by the European group and the GIMEMA. However, the results from the New York study, with median disease-free and overall survivals both greater than 57 months and 56% of the newly diagnosed patients without additional maintenance chemotherapy [76], and the GIMEMA study, with a two-year EFS of nearly 80%, compare well with early results of two-year EFS between 60% and 80% the European APL study.

Similarly, whether pulses of ATRA for 15 days every three months is sufficient or whether MP/MTX chemotherapy is of additional value requires ongoing study.

A humanized monoclonal antibody, M195, directed against CD33 has been used as consolidation for minimal residual disease in APL, with early success [86,100,101].

Prognostic factors

Older age, hemorrhagic diathesis on presentation, low platelet count, and elevated leukocyte count are poor prognostic factors for APL patients who receive ATRA therapy. The JALSG multivariate analysis showed that no or minor purpura at diagnosis ($p = 0.0046$) and age less than 30 years ($p = 0.0076$) were favorable factors for achievement of CR. Multivariate analysis showed that age less than 30 years ($p = 0.0003$) and initial leukocyte count less than 10×10^9/L ($p = 0.0296$) were prognostic factors for longer EFS, and initial leukocyte count less than 10×10^9/L was a sole significant factor for longer DFS ($p = 0.0001$) [84]. The European APL 91 study showed that, in the ATRA group, no factor was predictive of CR achievement: WBC greater than 5000/mm^3 ($p = 0.04$), platelets less than 50,000/mm^3 ($p = 0.03$), and circulating blasts greater than 500/mm^3 ($p = 0.007$) were associated with lower EFS; age

greater than 55 years ($p = 0.05$) and circulating blasts ($p = 0.03$) with shorter survival; platelets less than 50,000/mm^3 ($p = 0.02$) and circulating blasts greater than 500/mm^3 ($p = 0.004$) with higher incidence of relapse [88]. A British report showed that survival varied greatly by age: an 85% five-year survival for patients less than 25 years versus 24% for older patients ($p < 0.0001$). This result was largely due to a difference in early deaths by age: early death occurred in 1 of 34 patients less than 25 years versus 23 of 106 older patients ($p < 0.0001$). WBC greater than 10×10^9/L was also associated with poor prognosis ($p < 0.0005$). ATRA treatment improved survival only in the 25–39 year group ($p = 0.04$). On multivariate analysis, high WBC and age greater than 25 years were shown to be independent predictors of poor prognosis. The beneficial impact of ATRA within an unselected population was not shown [102].

APL may occur as a secondary leukemia [103–105]. As many as 12% of APL patients may have a history of another malignancy. Patients with secondary APL were significantly older (median age 56 vs. 36 years; $p < 0.01$) and had a lower incidence of hypofibrinogenemia ($p < 0.01$) than those with primary APL. The outcome of patients with secondary APL is similar to those with primary AML and should be treated in a manner similar to primary APL [103].

The effect of complexity of the karyotype has been analyzed, with conflicting results. In one study, the incidence of secondary cytogenetic abnormalities was 32% [106]. Secondary cytogenetic changes do not necessarily confer a poor prognosis in APL patients treated with anthracycline/cytarabine (Ara-C)-based chemotherapy [106]. However, in a series of patients from the Royal Marsden, 39% of patients (21 of 54) had additional chromosome abnormalities at presentation. In this series, the presence of additional chromosome abnormalities has an adverse effect on prognosis, independent of other prognostic indicators, reducing it to the level of patients with AML from less-favorable cytogenetic subgroups [107]. The presence of trisomy 8 did not worsen outcome, but patients with more complicated karyotypes had a trend toward reduced survival [102]. The incidence of secondary changes in the British series is almost 90%. In a German series, the incidence of secondary karyotypic abnormalities is 36% [108].

Complications of ATRA therapy

Compared to conventional chemotherapy, ATRA has been relatively safe. The most frequent side effects are those typical of retinoids, especially higher doses of vitamin A. Headache, with onset a few hours after ATRA ingestion, is the most common side effect and usually improves with continued therapy; pseudotumor cerebri, as seen with vitamin A toxicity, has occurred infrequently. Abnormalities of skin and mucous membranes include dry skin, pruritis, cheilitis, and xerostomia; bone pain and arthralgia, like headache, tend to occur early and improve despite continued ATRA treatment.

Leukemia cutis has been reported [109–111]. Mild hypercalcemia, hypertriglyceridemia and/or hypercholesterolemia, and transient elevations in liver enzymes also occur [29,61,66,87]. Retinoids have been reported to be teratogenic [112]. Therefore, pregnancy testing should be performed in women of childbearing age, and use of ATRA should be avoided in pregnant patients. However, use has been reported in the second and third trimester without obvious adverse effects [113–117]. Rare adverse effects include hypercalcemia, male infertility, bone marrow necrosis, fibrosis and acute pancreatitis, thromboembolic events, and RA-dependent growth of pathologic cells, including Sweet's syndrome, erythema nodosum, hyperhistaminemia, granulomatous proliferation, and mild cases of pulmonary complications [118,119]. Sinus bradyarrythymias have rarely been reported to be triggered by ATRA [120]. Hepatobiliary complications have also been reported [121]. Even with the combination of ATRA and chemotherapy, thrombotic complications were noted in 10% of patients during induction. Thrombotic events may be more common in patients suspected of having ATRA syndrome [122]. Pulmonary embolism has been reported [123].

The most important, and potentially fatal, toxicities are those of the *retinoic acid syndrome* (RAS) that occur in up to 25% of patients treated with ATRA monotherapy [124,125]. This syndrome includes fever, respiratory distress, pulmonary infiltrates, pleural effusions, and fluid retention. This syndrome may be fatal, with severe hypoxemia and multiorgan failure. Autopsies have shown extensive myeloid infiltration of lungs, liver, kidneys, lymph nodes, and skin [124]. Infiltration of the skin in the absence of other symptoms may occur for reasons parallel to the development of RAS [126,127].

This syndrome can occur at any time during the course of ATRA treatment but appears to be more common in patients with leukocytosis, especially those who develop leukocytosis (including recovery of normal white cells) on ATRA. However, one third of cases occur in patients with normal or low leukocyte counts. In the Intergroup 0129 study, the syndrome occurred after a median of 12 days of ATRA but ranged from day 2 to day 47. RAS was not observed during maintenance therapy with ATRA [125].

The etiology of RAS continues to be explored. Possible mechanisms include release of vasoactive cytokines, expression of adhesion molecules on myeloid cells, and migratory activity of differentiating APL cells [124,128,129]. In an in vitro model, ATRA-induced aggregation of maturing malignant promyelocytes is inhibited by LFA-1 and ICAM-2 MoAb and reversed by treatment with steroids [130].

Immediate recognition and treatment is essential for the survival of patients with the ATRA syndrome. Approaches to treatment have varied. Frankel et al. have described a high response rate with dexamethasone 10 mg intravenously every 12 hours for three days. The syndrome may recur and require further treatment [124]. An alternative approach has been the institution of chemotherapy to treat or prevent the ATRA syndrome. The French APL group added full-dose induction chemotherapy to ATRA for a white blood

cell count greater than 5000/uL on day 1, greater than 6000/uL on day 5, greater than 10,000/uL on day 10, or greater than 15,000/uL on day 15. This intervention resulted in 40%–65% of patients receiving chemotherapy during ATRA induction but has led almost to disappearance of the ATRA syndrome [63,72,73]. The Australian Leukemia Study Group evaluated prophylactic prednisolone, with chemotherapy added for rapid increases in WBCs; findings suggested a lower incidence of the ATRA syndrome in this small group of 25 patients [93]. Other interventions, including hydroxyurea and leukapheresis, are of unknown benefit.

The optimal management of the ATRA syndrome has not been defined. However, patients who develop evidence of the syndrome, especially those with fever, dyspnea, signs of fluid retention, and leukocytosis or rising WBC counts, should immediately receive dexamethasone and/or chemotherapy. It remains controversial whether to continue ATRA once the syndrome develops. Further drug administration may be useful if the syndrome occurs early to ensure that a sufficient period of retinoid exposure has been given to induce a remission. It has been the practice of the authors to continue ATRA with dexamethasone. In the North American Intergroup 0129 study, eight patients who developed RAS were continued on ATRA, with successful resolution of the syndrome. In patients in whom the ATRA was stopped, 90% of those who restarted had no additional problem. In patients who had ATRA stopped and not restarted, 50% entered a CR with 12–28 days of ATRA therapy prior to RAS [125]. Further understanding of the etiology and management of the ATRA syndrome should improve the therapeutic index of this drug. Lower doses of ATRA have been suggested to result in a lower frequency of patients with RAS, but doses of 25 mg/m^2 in the absence of administration of cytotoxic chemotherapy still resulted in RAS developing in some patients [43,44].

ATRA is associated with unusual sites of relapse. By upregulating intercellular adhesion molecules, ATRA may facilitate the passage of malignant promyelocytes across the blood–brain barrier and result in an increase in extramedullary relapses [131,132]. CNS relapse has been reported [133,134]. Extramedullary relapses have also been reported in the external auditory canal [135].

Costs of ATRA therapy

The average wholesale price (AWP) of ATRA to pharmacies in the United States is approximately $1000 per 100 capsules or $10 per capsule. For an adult requiring 80–100 mg/day, the wholesale cost will be $80–$100; obviously, the charge to patients will be higher. The impact of ATRA on the costs of induction therapy for APL depends less on the cost of the drug than on its potential to decrease the length of hospitalization and to decrease the need for intensive supportive care for hemorrhagic and infectious complications associated with conventional chemotherapy. Eardley et al. compared the hospital costs of

initial remission induction with ATRA (median $61,756) to those of conventional chemotherapy ($97,944) in sequential patients with untreated APL [136]. ATRA induction therapy was associated with significant decreases in hospital days, platelet and red cell transfusions, and median days of combination antibiotics and amphotericin [136]. A cost analysis performed in Japan showed a difference in hospital bills between $46,300 in the chemotherapy group and approximately $32,300 in the ATRA group ($p < 0.0001$). The difference resulted mainly from the costs of antibiotics and of platelet and erythrocyte transfusions [137]. A recent abstract adds support to these findings; median charges for patients who have received ATRA for APL were 65% or less of charges for patients with other subtypes of AML [138]. However, ATRA's greatest impact on decreased costs may result from the improvements in disease-free and overall survival, avoiding the high costs associated with reinduction therapy for patients with relapsed refractory APL and with supportive care for patients with terminal disease.

Other retinoids

Although the bulk of clinical trials of retinoids in APL have used tretinoin, other retinoids have been shown to be effective in patients with APL. The naturally occurring 9-*cis* retinoic acid binds to both RAR and RXR, has been shown to be effective in NB4 cells, and has been tried in a limited number of patients [139–143]. Two synthetic retinoids namely, Am80 and Am580, have demonstrated efficacy in relapsed patients [144]. The hope is that these new retinoids will have comparable or superior clinical activity to ATRA and can overcome ATRA resistance.

Am580, a stable retinobenzoic derivative originally synthesized as an RARα agonist, is a powerful inducer of granulocytic maturation in NB4, an APL-derived cell line, and in freshly isolated APL blasts at concentrations that are 10- to 100-fold lower than those of ATRA necessary to produce similar effects. Am580 is more powerful than ATRA in modulating the expression of differentiation antigens only in cells in which PML-RAR is present [145]. This finding suggests that the mechanism of the therapeutic effect of retinoids is mediated through the aberrant transcript PML-RAR, likely by the binding of the ligand removing a dominant negative inhibition of normal differentiation of the promyelocyte. The synthetic retinoid, Am80, is approximately 10 times more potent than ATRA as an in vitro differentiation inducer; is more stable to light, heat, and oxidation than ATRA; has a low affinity for cellular retinoic acid binding protein; and does not bind to RARγ. In APL that had relapsed from CR induced by ATRA in a prospective multicenter study, patients received Am80, 6 mg/m^2, orally alone daily until CR. Of 24 evaluable patients, 14 (58%) achieved CR. The interval from the last ATRA therapy was not different between CR and failure cases. The clinical response was well correlated with the in vitro response to Am80 in patients examined. Adverse events

included 1 retinoic acid syndrome, 1 hyperleukocytosis, 9 xerosis, 8 cheilitis, 16 hypertriglyceridemia, and 15 hypercholesterolemia, but generally milder than those of ATRA, which all patients had received previously. Am-80 is effective in APL relapsed from ATRA-induced CR and deserves further trials, especially in combination with chemotherapy [146,147]. Other RAR-specific agents are undergoing development.

Another approach has been to modify tretinoin by placing it within a liposome. Liposomal ATRA (L-ATRA) has been shown to induce remissions in de novo and relapsed APL patients and continues to undergo clinical evaluation [148]. This compound is administered intravenously and may have pharmacokinetic advantages over oral ATRA.

Arsenic

Arsenic compounds have been in use in China and were reported to show a strong therapeutic effect against APL. A series of patients treated with arsenic trioxide in relapse has prompted more formal investigation of this compound, both in the lab and in the clinic [149]. As_2O_3 can induce clinical remission in de novo or relapsed APL patients and has no cross-resistance with ATRA. It has dual effects on APL cells, namely, preferential apoptosis at high concentration (0.5–2 μM) and partial differentiation at low concentration (0.1–0.5 μM) [150]. Modulation and degradation of PML-RARα proteins can be induced by As_2O_3 and probably contribute to these two effects. These studies lead to a model in which PML-RARα could be the target of both ATRA differentiation therapy and As_2O_3 apoptosis therapy [151]. Among the clinical leukemia samples tested, the in vitro cytotoxic effects of As_{3+} were observed selectively in APL cells, regardless of their ATRA sensitivity. These data suggest that APL cells are sensitive to arsenic Sp and that arsenic Sp acts on APL cells via a different pathway to ATRA [152].

Among a group of 15 APL patients who had relapsed after receiving ATRA-based therapy, clinical CR was achieved in 9 of 10 (90%) patients treated with As_2O_3 10 mg/day intravenously alone and in the remaining five patients treated by the combination of As_2O_3 and low-dose chemotherapeutic drugs or ATRA. Bone marrow suppression was not observed. Arsenic levels showed that after a peak level of 5.54–7.30 μmol/L, plasma arsenic was rapidly eliminated, and the continuous administration of As_2O_3 did not alter its pharmacokinetic behavior. A decline of the arsenic content in hair and nail was observed after withdrawal of the drug [149].

Stem cell transplants

Because some patients still relapse after ATRA plus chemotherapy, prognostic factors for relapse need to be precisely determined. These certainly include persistence or reappearance of PML-RAR transcript during follow-up. Allogeneic BMT should be considered in those patients. With the exceptional

overall outcome of induction and consolidation therapy, stem cell transplant should be reserved for either molecular persistence or relapse or after clinical relapse. In patients without a suitable family donor, autologous stem cells could be procured in first remission for potential use at relapse.

The outcome of autologous transplant for APL in second remission may be predicted by RT-PCR status. Patients who remain PCR+ after transplant relapse early, while most patients who remain PCR− remain in remission. PCR+ patients are candidates for investigational therapy or allogeneic transplant [153].

Antileukemic action of the cytoreductive conditioning regimen may convert patients who are PCR positive to negative and lead to durable remissions [154].

Ligand–receptor interactions, transcriptional regulation, and targeted transcription therapy

The retinoic acid receptor alpha (RARα) is one of a family of three nuclear retinoic acid receptors [155,156]. The gene that encodes the RARα is located on the long arm of chromosome 17 [157] and is translocated to chromosome 15 in APL cells forming a fusion with the PML gene [158]. This PML/RARα fusion product is diagnostic of APL and appears to be critical to leukemogenesis [159–163]. Proof of the principle that this fusion protein is the pathogenic marker of the disease has been demonstrated by examining mice harboring a PML/RARα transgene whose expression is specifically targeted to the myeloid–promyelocytic lineage. These mice develop acute myeloid leukemia with promyelocytic features [164]. However, four fusion partners with RARA have been demonstrated in APL: PLZF, NPM, and most recently the nuclear mitotic apparatus protein NuMA. NPM-RARA APL differentiates in vitro to ATRA, whereas PLZF/RARA APL may be clinically resistant [165].

Rare resistant patients who have been studied and RA-resistant NB4 APL cell lines have provided the opportunity to define the mechanism of ATRA's ability to induce terminal differentiation of the malignant promyelocyte [162]. In RA-resistant subclones of the human APL cell line, NB4, whose nuclear extracts display altered RA binding, absence of ligand binding of PML-RARα is associated with a point mutation in the ligand-binding domain of the fusion PML-RARα protein. The mutant PML-RARα protein retains the ability to heterodimerize with RXRα and thus to bind to retinoid response elements (RAREs). This leads to a dominant negative block of transcription from RAREs that is dose-dependent and not relieved by RA [162]. Moreover, the functional analysis of the various fusion proteins, as well as RARα partners, is revealing striking common features beneath a misleading structural heterogeneity that unravels a possible unifying molecular mechanism towards APL leukemogenesis [161]. The heterogeneity of the partner proteins and the varia-

tion of response of these molecular variants to retinoic acid suggest that interference with retinoid signaling, and not disruption of PML organization, is essential to the APL phenotype [166].

APL cells become resistant to RA-induced differentiation both in vitro and in patients. Although pharmacodynamic mechanisms of resistance have been reported, there is growing evidence that resistance both in patients and in vitro can be mediated by changes in the sensitivity of leukemic cells to retinoids. These changes may involve altered ligand binding patterns of PML/RAR that impede the ability of retinoids to bind to and activate RAREs [167]. The role of the PML/RARA as the target of RA action is further substantiated by the identification of mutations in PML in RA-resistant APL cell lines [168]. Differentiation of APL by RA is triggered directly through PML-RARα and is associated with its degradation [168].

Normal RARs repress gene transcription by associating with ancillary factors, denoted *corepressors*. The fusion oncoproteins PML-RARα and PLZF-RARα retain the ability of RARα to associate with corepressors, and this corepressor association correlates with certain aspects of the leukemic phenotype. This finding suggests that transcriptional silencing by the fusion protein may be the method of pathogenesis and also that the addition of retinoic acid may reverse a dominant negative repression by interacting with the fusion proteins [169]. Recently, the oncogenic fusion proteins that pair with RAR have been shown to interact with the nuclear receptor corepressor N-CoR [148,170–172]. Pharmacologic concentration of ATRA could dissociate N-CoR from PML-RARα and allow for removal of the dominant negative suppression of normal promyelocyte development. This repression complex also involves a histone deacetylase complex [148].

With the knowledge that the transcriptional repressor ability of RARA fusion proteins is the main leukemogenic mechanism of APL, nuclear receptor transcriptional corepressors, such as SMRT and histone deacetylases, are now targets for therapy in APL [148,170–172]. The formation of PLZF-RARα, but not PML-RARα, corepressor complexes that are insensitive to RA through the PLZF moiety now explains the retinoid resistance of the t(11:17) variant APL. Histone deacetylase inhibitors such as Trichostatin A (TSA), in combination with RA, can overcome the transcriptional repressor activity of PML-RARα and PLZF-RARα as well as the unresponsiveness of PLZF-RARα-expressing leukaemic cells to RA [170].

Conclusion

APL has become the model for understanding the pathogenesis of leukemia and has been successfully treated with therapies that are disease specific. Further investigation of the molecular pathogenesis of this disease will allow additional therapeutic advances. Continuing improvements in clinical management of APL offer a chance for cure.

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6. Prognosis in multiple myeloma

Rafael Fonseca and Philip R. Greipp

Each year nearly 15,000 patients are diagnosed with multiple myeloma in the United States alone, and most of them will die of disease progression [1]. For physicians to be able to discuss natural history with patients and tailor therapeutic modalities according to expected outcome, accurate prognostic systems are needed. Although several factors have been found to be prognostic in myeloma, there is no definitive consensus on which combination of these factors should be used. Accurate prognostication is important in everyday clinical practice as well as in the design of clinical trials. In this chapter, we will try to discuss some of the most relevant prognostic factors currently used.

A wide spectrum in survival is seen among patients with multiple myeloma, ranging from a fulminant course lasting only weeks to months to a more indolent course with survival in excess of five years [2]. At present we are unable to precisely identify both groups at diagnosis. There are some patients for whom early aggressive treatment may be recommended due to poor prognostic features, while others may benefit just as well from conventional chemotherapy, due to their longer expected survival. Multiple myeloma is a complex disorder, or group of disorders, for which no simple classification system can reliably predict the expected outcome.

We review here both clinical and laboratory features that are thought to have biologic and prognostic relevance in patients with myeloma.

Prognosis

An attempt to classify myeloma prognostic factors into three categories is currently favored: characteristics unique to the malignant clone; features reflecting or affecting tumor burden, including cytokines; and host features. We therefore will present our summary in the same order.

Characteristics of the malignant clone

Plasma cell labeling index. The plasma cell labeling index (PCLI) is one of several assays that reflect the proliferative activity of the monoclonal plasma

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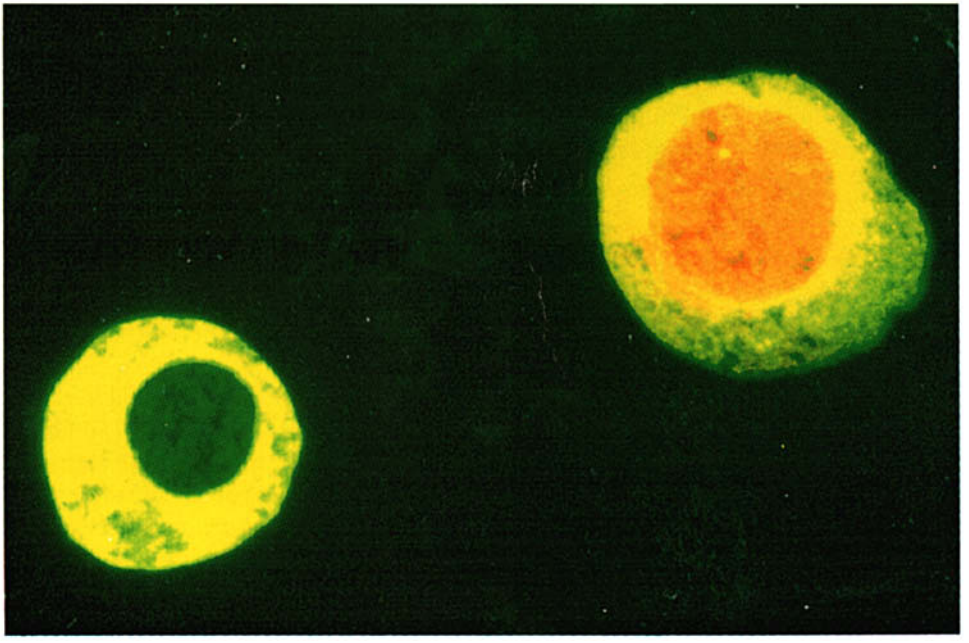

Figure 1. Plasma cell labeling index assay. Plasma cells are distinguished from other hematopoietic elements by the fluorescence of the cytoplasmic immunoglobulin. The plasma cell on the right is counted as positive, since the bromodexyuridine is recognized in the nucleus. (Courtesy of Dr. Philip R. Greipp.)

cells. The study is an immunofluorescent slide-based assay [3]. Cells in the S phase of the cell cycle incorporate bromodeoxyuridine, which can be recognized by a specific fluorescent monoclonal antibody directed against it (rhodamine conjugated) (Figure 1). Plasma cells are marked and recognized by a kappa-or lambda-specific anticytoplasmic immunoglobulin monoclonal antibody (fluorescein conjugated). The percentage of plasma cells in S-phase is the PCLI [3]. The PCLI provides an indication of plasma cell proliferation.

There is ample evidence to support the use of the PCLI as a prognostic factor in myeloma patients. An elevated PCLI predicts short remission and survival. Separate prognostic studies done at the Mayo Clinic and elsewhere using a Cox proportional hazards model have confirmed the independent prognostic capacity of the PCLI assay [4–11] (Table 1). Boccadoro and colleagues have reported that the prognostic value of the PCLI is especially useful among rapid responders to chemotherapy [7]. They found that among rapid responders the median survival of those with a high labeling index ($\geq$2%) was much shorter than that of patients with low values (<2%) (16 months versus $\geq$47 months). The short survival of patients with a high PCLI was due to shorter remission duration. At the end of two years, nearly all

Table 1. Prognostic value of plasma cell labeling index (PCLI)

Author	Year	Cases	PCLI %	Median survival (mos.)	Univariate *p*-value
Durie	1980	79	≥1%	NA	0.02
			<1%	NA	
			≥3%	NA	0.002
Latreille	1984	50	≥1%	NA	0.04
			<1%	NA	
Montecucco	1986	37	≥2%	10	<0.01
			<2%	55	
Greipp	1987	100	≥0.8%	18	0.02
			<0.8%	38	
Boccadoro	1989	65	≥2%	16	<0.004
		42	<2%	47	
Boccadoro	1991	173	≥2%	16.4	<0.0001
			<2%	42.2	
Greipp	1992	419	≥2%	21.4	<0.001
			<2%	42.7	
Greipp	1993	107	≥1%	17	0.0004
			<1%	42	
			≥2%	15	<0.0001
			<2%	40	
Cornelissen	1994	63	≥3%	17	0.029
			<3%	29	

From Greipp and Kyle. 1996. Staging, kinetics, and prognosis of multiple myeloma. In: Wiernik PH, Canellos GP, Dutcher JP, Kyle RA (eds), Neoplastic Diseases of the Blood, 3rd ed. Churchill Livingstone. New York, NY 1966.

patients with a high PCLI had died. Most investigators use a cutoff value between 1% and 2% to identify those with a poor prognosis.

Other proliferation assays. Other methods are available to evaluate the proliferative activity of the plasma cells. Immunohistochemical staining of bone marrow for detection of proliferating plasma cells using the Ki-67 antibody has been proposed as a surrogate marker for the PCLI, because it also identifies the percentage of plasma cells in active cell division [12]. The correlation between the results of this assay and disease activity is good, but comparisons suggest a wider variation of results and lack of correlation with the bromodeoxyuridine assay [13].

Similarly, the use of the proliferating cell nuclear antigen (PCNA) monoclonal antibody [14] and percentage of plasma cells in S-phase [15,16] have been proposed as a surrogate marker of cell proliferation. The results of PCNA correlate more closely with PCLI that the Ki-67 assay does. The S-phase determination through DNA content flow cytometric analysis of Ig-positive cells is restricted in its use for patients with 20% or more plasmacytosis in the bone marrow [16]. San Miguel and colleagues analyzed the cell cycle distribution of plasma cells in 120 untreated multiple myeloma patients using simultaneous CD38 staining [15]. They found that the percent-

age of S-phase cells in the myeloma population was found to be significantly lower than that of the residual normal bone marrow cells ($p < 0.001$). On multivariate analysis, they showed that the number of S-phase plasma cells was the most important independent prognostic factor. They then constructed a model where the percentage of S-phase plasma cells, in conjunction with beta 2-microglobulin (B2M), age, and performance status, allowed accurate classification of patients into different prognostic groups.

Interpretation of PCNA and Ki-67 antibody assays and the S-phase percentage is complicated by the difficulty in the positive identification of malignant plasma cells and exclusion of more proliferative marrow elements from the assay results. Further validation and testing of these techniques need to be done before they can be adopted.

Cytogenetic abnormalities and DNA content. Multiple and complex chromosomal abnormalities have been described in patients with myeloma. The frequency of observed abnormalities with conventional cytogenetics is low (19%–30%) [17–20]. Unfortunately, standard cytogenetic evaluation is limited and can be improved by newer, more sophisticated and sensitive techniques.

PLOIDY. Myeloma plasma cells almost always have an abnormal DNA content as determined by flow cytometric ploidy analysis; however, studies have suggested that ploidy has a modest effect in survival [21]. A negative prognosis has been suggested for patients whose tumors are hypodiploid [21]. However, a recent analysis of study E9486 (ECOG) failed to confirm the poor prognosis associated with the hypodiploid state [22]. Furthermore, the main limitation of the hypodiploid state as a prognostic factor is that only about 1%–5% of patients have this finding.

PROGNOSTIC SIGNIFICANCE OF CONVENTIONAL CYTOGENETICS. Recently, Tricot and colleagues have found that the presence of unfavorable cytogenetics is independently associated with a poor outcome in patients undergoing high-dose chemotherapy for the treatment of multiple myeloma [23,24]. Our group and others have confirmed that the presence of abnormal cytogenetics, as determined by conventional cytogenetics, is associated with an adverse outcome in these patients [25]. However, interpretation of these reports is limited because of the problems inherent to conventional cytogenetics. We have recently found a significant correlation between the presence of cytogenetic abnormalities and a high PCLI (S.V. Rajkumar et al., unpublished observations). This finding confounds the interpretation of abnormal cytogenetics, determined conventionally, for prognostication in myeloma. The independent prognostic significance of conventional cytogenetics in patients receiving conventional chemotherapy is not currently known.

Among the many difficulties in the evaluation of myeloma, conventional cytogenetics depends on the proliferation of the cells examined, and relative to

normal marrow elements, malignant plasma cells divide slowly. Resulting metaphases may or may not originate from the malignant clone. Therefore, results of conventional cytogenetics are biased, because even if metaphases are obtained from plasma cells, they may not be representative of the myeloma clone. Similarly, the presence of abnormalities in conventional cytogenetics may be a mere marker of a higher proliferative state of the malignant plasma cells in some cases. Because the frequency of numerical abnormalities, including monosomy and trisomy, is unknown, their specific prognostic significance is currently unknown.

The use of interphase FISH analysis has revolutionized the diagnostic capabilities for analyzing chromosomal abnormalities in slowly dividing malignant cells. Using interphase FISH, our group and others have determined that numerical chromosomal abnormalities are frequently seen in myeloma, even in those cases deemed 'normal' by conventional cytogenetics [26–32]. We have refined the standard FISH technique by incorporating simultaneous fluorescent staining of the cytoplasmic immunoglobulin (cIg-FISH) [33]. With this addition, we can precisely identify plasma cells and perform cell-specific FISH analysis. In this way, the distinction between plasma cells and other hematopoietic cells can be done confidently.

IMPORTANCE OF CHROMOSOME 13 ABNORMALITIES. The presence of partial or complete deletions of chromosome 13 using FISH analysis has been associated, in a retrospective fashion, with a poor prognosis in myeloma patients [34]. Whether this association is secondary to deletions of the *Rb* gene, located in chromosome 13, is currently unknown. Deletions of the *Rb* gene are common in myeloma [35], and loss of this tumor suppresser gene may allow IL-6 upregulation [36]. The *Rb* gene plays a crucial role in cell cycle regulatory mechanisms. In its active state, *Rb* is not phosphorylated, and it prevents the passage of the cell into an active mitotic stage. When phosphorylated, it becomes inactive and permits cell cycle progression. Deletions of this gene product may correlate with a higher proliferative state, and thus could be associated with a poor prognosis in neoplasias.

IMPORTANCE OF 11Q13 TRANSLOCATIONS. The presence of structural abnormalities of chromosome 11q13 could be of prognostic significance, as has been suggested by Tricot and colleagues [23,24]. In our experience, this translocation is the most common one seen in myeloma [17]. The region 11q13 hosts the *cyclin D1* gene, which may be overexpressed when juxtaposed to the 14q32 heavy chain promoter region [37]. When *cyclin D1* is overexpressed, cell cycle progression is favored through phosphorylation of the *Rb* gene product. Since the aforementioned limitations to conventional cytogenetics apply for this analysis, interphase FISH could be useful to validate this contention. Through the use of interphase FISH and fiber-FISH, structural chromosomal abnormalities will hopefully be better defined in myeloma patients [38,39].

Oncogenes and tumor suppressor genes. Several mechanisms may be involved in the dysregulation of oncogenes/tumor suppresser genes in myeloma pathogenesis. Oncogenes may be overexpressed, frequently as a result of chromosomal translocations. The activity of tumor suppressor genes may be lost due to deletions, mutations, or methylation.

Mutations in the oncogene *ras* have been noted in plasma cells of myeloma, more commonly in the advanced phases of the disease [40,41]. The prognostic capacity of *ras* abnormalities is being currently defined. Liu, Van Ness, and colleagues examined the mutational status of the N- and K-*ras* genes in 160 newly diagnosed myeloma patients enrolled on the Eastern Cooperative Oncology Group (ECOG) phase III clinical trial E9486 [42]. The incidence of *ras* mutations among the samples analyzed was 39%. Patients with K-*ras*, but not N-*ras*, mutations had a significantly shorter median survival (2.0 vs. 3.7 years, $p < 0.02$) [42].

The *c-myc* gene is frequently overexpressed in myeloma but usually without evidence of rearrangements [43,44]. Although the mechanism for *c-myc* overexpression is not known, it might play a role in early myeloma development.

Mutations of the tumor suppresser gene p53 have also been observed and are more common in the late stages of the disease. Neri and colleagues have described an incidence of 13% point mutations in myeloma patients (7 of 52) [45]. In this study, the mutations were more common in the more advanced and clinically aggressive/leukemic form of the disease (43%, 7 of 16 patients). A prognostic value for p53 deletions as determined by FISH has been recently reported by Drach and colleagues [46]. They found that p53 interstitial deletion can be seen in 30% of newly diagnosed myeloma patients and predict for short survival in those treated with conventional chemotherapy. Further studies to determine the nature of p53 abnormalities and the relations they hold to disease pathogenesis are needed.

Plasma cell morphology and immunohistochemistry. In 1948, Bayrd showed that patients with myeloma that had very immature morphologic features in the bone marrow had a shortened survival. It was not until the 1980s that this observation could be confirmed [47]. Studies done at our institution and elsewhere have confirmed the association between plasmablastic morphology and shortened survival [47–49]. Of 453 patients enrolled in E9487, 37 were independently confirmed as plasmablastic morphology by two reviewers. Plasmablastic morphology correlated with known predictors of survival, including significantly lower hemoglobin and serum albumin, higher calcium and B2M levels, and higher percentage of bone marrow plasma cells by immunofluorescence. Patients with plasmablastic morphology had higher PCLI, higher serum-soluble interleukin-6 receptor (sIL-6R) levels, and a higher probability of *ras* mutations . In a univariate analysis, the median survival for the plasmablastic group was 1.9 versus 3.7 years for the nonplasmablastic group ($p < 0.0001$). It has been suggested that the plasmablastic morphology

is not associated with an unfavorable outcome unless another group of the so-called immature subtype is included [50]. One limitation of plasmablastic morphology as a prognostic factor is that interpretation is necessarily subjective, and reproducibility may be limited.

Immunohistochemical studies have suggested an adverse outcome for patients with elevated levels of acid phosphatase and β-glucuronidase [51,52]. Findings of immaturity on electron microscopy have also been associated with a poor prognosis [53].

Phenotyping. Phenotypic characterization of myeloma cells by flow cytometric analysis and immunocytochemical stains demonstrates abnormalities in varying percentages of patients. Although most patients express mature plasma cell surface antigens, such as CD38 or PCA-1 [54], some express immature lymphocyte antigens such as CD10 and CD20 [55]. Groups of patients have been classified into good or poor prognosis groups according to the expression of CD10 [56,57]. There is evidence that expression of CD20, a late B-cell but pre-plasma-cell antigen, may correlate with poor prognosis [58]. We have been unable to confirm this association in the patients enrolled in E9487 (Philip R. Greipp, unpublished observations).

Features that reflect tumor burden and cytokine activity

β2-Microglobulin. β2-Microglobulin (B2M), first studied by Bataille and associates, has been a reliable predictor of survival in myeloma patients [59,60]. Results of studies done with B2M have shown that it predicts for early death but does not predict response to chemotherapy; normal values predict a long median survival. Repeated studies have validated its prognostic capacity. When used in conjunction with the PCLI, a simple and powerful prognostic model can be used (Figure 2) [6]. Young patients (<63 years) with low PCLI and low B2M have a median survival of over six years. Therefore, these patients may benefit by conventional chemotherapy and not be subjected to high-dose chemotherapy with stem cell support until later in their disease course. Using landmark analysis, the British Medical Research Council found that after two years of survival, the initial B2M loses its prognostic capability [61].

Although B2M is a powerful predictor of tumor burden, other factors affect its concentration as well. It is a small molecule of 19 kDa cleared by the kidneys. Therefore, its concentration is elevated in renal failure states. There is no accepted way of correcting for a given degree of renal insufficiency [62]. B2M is a highly significant prognostic factor in myeloma patients with normal renal function.

IL-6. The search for an essential growth factor in myeloma culminated in the identification of IL-6 as the formerly known B-cell growth factor or hybridoma

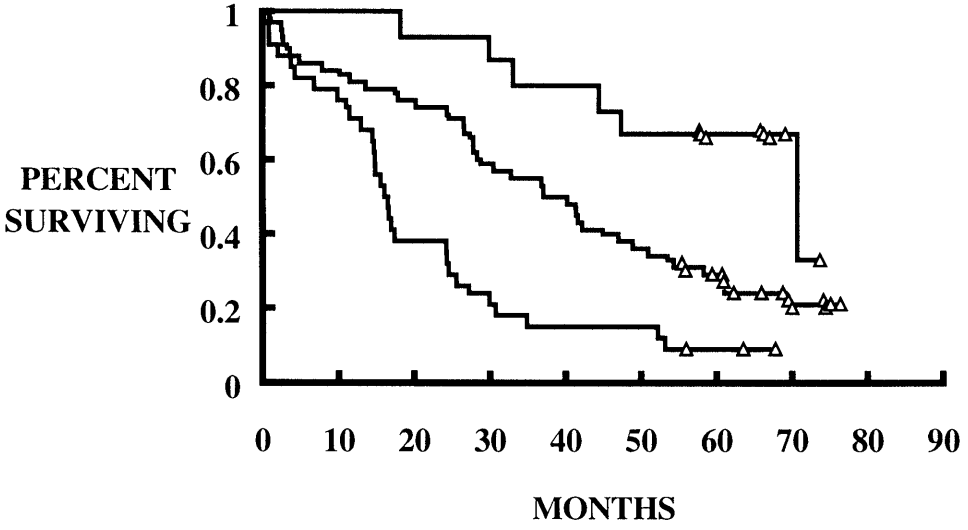

Figure 2. Kaplan–Meier overall survival curves for myeloma patients grouped by plasma cell labeling index and $\beta_2$microglobulin (B2M). The upper curve represents patients with a low B2M (<2.7 μg/mL) and a low labeling index (<1%) (n = 15). The lower curve represents those patients with a high B2M (>2.7 μg/mL) and high labeling index (>1%) (n = 34). Patients with either of these measures elevated are depicted in the middle curve (n = 58). (From Greipp [6], with permission from author.)

growth factor. Because of the positive effects of IL-6 in myeloma plasma cells, a prognostic use of the cytokine level has been proposed. To determine whether IL-6 or surrogate markers predict for survival or freedom from relapse, several studies have been performed. Serum levels of IL-6 have been shown to have prognostic value in myeloma [63]. It is known that serum IL-6 correlates with bone marrow plasmacytosis, serum LDH, B2M [64], and calcium [65]. It also correlates inversely with hemoglobin level. More advanced stages of myeloma have higher concentrations of serum IL-6 [64]. These and other observations suggested a correlation between serum IL-6 and tumor mass. The results of several studies have been inconsistent, probably due to methodological differences.

C-reactive protein. Observations by Bataille [66] and others [67] showed that C-reactive protein (CRP), a surrogate marker for IL-6, is predictive of survival in myeloma patients. Our group confirmed the univariate analysis significance of CRP as a predictor of prognosis in a group of myeloma patients [67]. However, the multivariate analysis showed that once PCLI and B2M are known, CRP does not add further information to the model. These findings may have been related to a low sample size. Recent studies done at ECOG have failed to confirm the independent value of CRP as an independent predictor of prognosis [48,68].

Table 2. Combination of PCLI, B2M, and sIL-6R predict survival in myeloma

PCLI >2%, B2M >4, and sIL-6R >300	No.	Estimated two-year survival	Median survival
2,3 values high	65	41%	18mos.
1 value high	150	75%	37mos.
0 values high	173	83%	Not reached

From Greipp PR [88], with permission from author.

Soluble IL-6 receptor. Soluble IL-6 receptor (sIL-6R) has been found to be an independent predictor of survival [67]. We analyzed sIL-6R in 420 patients who received VBMCP-based chemotherapy and with a median follow-up time of 24 months. Using the Cox proportional hazards model, higher levels of serum sIL-6R were associated with shorter survival. A multivariate analysis of 388 cases with complete information showed independent prognostic significance for an sIL-6R greater than 300. The sIL-6R level did not correlate with PCLI or other covariates [67]. Adding sIL-6R to PCLI and B2M allowed improved prognostic classification, doubling the proportion identified as high risk (Table 2). Mutant sIL-6 receptor constructs, inhibitory to myeloma cell growth, may be tested for antimyeloma activity.

Other cytokines. An elevated IL-2 level has been suggested as associated with a better prognosis [69]. Because the source of this IL-2 may be activated T cells, it is intriguing that treatment with IL-2 might lead to successful therapeutic intervention.

Serum markers. High levels of serum lactic dehydrogenase (LDH) have been associated with an aggressive, lymphoma-like presentation of the disease [70]. Bulky retroperitoneal adenopathy and a short survival characterize this uncommon variant of myeloma. The proportion of patients with an elevated serum LDH ranges between less than 5% to 11%, depending on the population studied [71]. The prognostic capacity of LDH is thus limited because of the small number of patients with elevated levels of this serum marker.

Serum levels of thymidine kinase have been shown to have prognostic value in myeloma [72]. Thymidine kinase is independent of B2M and correlates partially with PCLI. In our experience, thymidine kinase serum levels identify only a small subset of the patients who have an elevated PCLI [67]. In a study by the British Medical Research Council (MRC), high levels of thymidine kinase levels were prognostic in patients treated with melphalan but not for patients treated with doxorubicin, carmustine, cyclophosphamide, and melphalan.

Recently Seidel, Børset, and colleagues have determined that elevated serum concentrations of hepatocyte growth factors in myeloma may identify a subgroup of patients with poor response to melphalan and prednisone chemotherapy [73]. On univariate analysis, hepatocyte growth factor was a significant

predictor of survival, but when multivariate analysis was performed it lost its predictive capacity. On subset analysis, it was also noted that elevated serum level of hepatocyte growth factor was of independent prognostic significance in patients with high serum B2M.

An association between high serum levels of type I collagen (ICTP) and survival has been suggested in newly diagnosed myeloma patients [74]. In one study, patients with high serum level of ICTP had a median survival of 3.5 years as compared to patients with low levels who had a median survival of 4.1 years ($p = 0.001$) [75]. The role of other serum markers such as serum neopterin [76], AAT [77], and soluble NCAM [78] is interesting and needs to be further investigated.

Circulating plasma cells. Circulating myeloma cells are difficult to find in routinely stained peripheral blood slides of most patients with myeloma [79]. However, with the use of multiparameter flow cytometry, these cells are more easily detected and quantified. Using a large sample size and stringent statistical criteria, Witzig and colleagues have defined that the presence of large numbers of circulating plasma cells, as determined by the peripheral blood labeling index, correlates with poor prognosis [80]. In their study of 254 patients, they found that the median percentage of circulating monoclonal plasma cells (among cIg-positive cells) was 6% and that 57% (144 of 254) of patients had a high number ($\geq$4%) of these cells. The latter group had a median survival of 2.4 years as compared to 4.4 years for those with less than 4% ($p < 0.001$). In a multivariate analysis, the percentage of circulating monoclonal plasma cells, age, albumin, stage, bone marrow PCLI, and the peripheral blood labeling index were independent factors for survival. The percentage of circulating monoclonal plasma cells and the bone marrow PCLI best separated the group into low-, intermediate-, and high-risk myeloma, with median survivals of 52, 35, and 26 months, respectively.

Host factors

Age and performance status. Age, performance, and socioeconomic status will have an influence on the outcome of patients with myeloma. Readily available clinical features such as hemoglobin concentration, creatinine, calcium, albumin level, immunoglobulin class subtype, and bone marrow degree of involvement can be used as predictors of survival. The prognostic classification of these variables has been refined by incorporation into the Durie–Salmon staging system [81]. Unfortunately, this system has limitations and has occasionally failed to predict survival [82]. Prognostic systems based on the prospective trial E9487 have failed to confirm an independent predictive independent predictive capacity for the Durie–Salmon staging system [48]. The quantification of bone lesions is not always reliable [82]. Therefore, better staging systems are needed.

It has been proposed that, according to the age of the patient, one can choose which treatment modality is more adequate. It follows that for the young patient one should consider the use of a high-dose chemotherapy regimen, while for the elderly the use of conventional chemotherapy is more appropriate. It is for those patients between these boundaries that the prognostic factors may be critical in the choice of optimal treatment. Unfortunately, no prospective randomized information exists to support the use of this classification. While it seems logical at first glance, most people would recognize that there are some young patients for whom expectant observation would be more appropriate, while an occasional 'elderly' patient may be a suitable candidate for high-dose chemotherapy.

Regulatory cells. Because the immune status of the host is thought to play a role, San Miguel and colleagues have demonstrated that loss of CD4-positive T cells is associated with a worse prognosis in myeloma patients [58,83]. Similarly, elevated level of T cells with activation markers or plasma cell markers has been associated with an unfavorable outcome [84]. Recent evidence has suggested the existence of idiotype-specific T cell clones in the peripheral blood of patients with plasma cell disorders [85,86]. The prognostic value of this finding is not yet confirmed. Recent analyses of circulating cells in myeloma by Kay and colleagues showed that approximately 20% of patients had increased levels of circulating CD19+ B cells [87]. Low numbers of circulating CD19+ B cells ($<125\,\mu L$) were associated with more advanced disease (clinical stage III, $p = 0.033$). Survival was better in patients with higher numbers of circulating CD19+ cells ($\geq 125/\mu L$, $p < 0.0001$), and CD19+ values were found to be independently prognostic [87].

Use of prognostic information and future directions

Prognostic information is of importance to both the patient and the physician. It may be critical in the choice of appropriate therapeutic modalities. It is also necessary for comparison between treatment arms in prospective randomized trials. As stated before by San Miguel, most patients' prognosis is currently determined on the basis of age, clinical performance status, and the Durie–Salmon clinical staging system. This determination is far from perfect, since the staging system measures primarily tumor burden. Furthermore, an improved staging system is needed for stratification in clinical trials, where patients are currently stratified for analysis using individual prognostic factors, with little attention paid to possible interaction between these factors.

Many prognostic factors are interrelated, and therefore choosing the best combination between them is complicated. However, the best combination thus far described includes B2M as a marker of tumor burden and cell proliferative activity (by either the labeling index or the number of plasma cells in

S-phase). Ultimately a combination of a measurement of tumor burden and a marker of the rate of increment of the monoclonal plasma cell population may be the best approach. In addition to the labeling index as a marker of proliferation, other features such as apoptotic rates may be important in determining the increment in the monoclonal plasma cells. After prognostic factors are deemed to be independent predictors, they should then be tested in combination to assure greatest accuracy.

Currently available prognostic factors can only be considered preliminary. Comprehensive studies linked to prospective randomized trials may be able to answer objectively some of our current questions. As novel emerging factors and detection techniques are described, they may replace these others in the evaluation of myeloma patients. For instance, cytogenetics is giving rise to important prognostic factors. These may eventually provide a rational basis for the subclassification of myeloma into biologically similar subgroups.

Conclusions

Although great progress has been made in our understanding of myeloma, better prognostic factors are needed. Through continued efforts in the basic, translational, and clinical research arenas, this goal seem achievable. Ultimately, the use of prognostic factors will enable better service to our patients.

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7. Bone marrow and stem cell transplantation for multiple myeloma

David H. Vesole

Multiple myeloma is an incurable B-cell malignancy afflicting mainly elderly people with a median age of 65 to 70 years [1]. It is characterized by the proliferation of clonal plasma cells that are resistant to standard doses of commonly employed agents at the time of diagnosis, resulting in complete remission (CR) rates of only 5%–10% and median survival of approximately three years. This inherent resistance is probably due to an array of molecular abnormalities of one or more oncogenes and suppressor genes inhibiting drug-induced apoptosis, including ras, myc, p53, Rb, and bcl-2, that have evolved during a prolonged subclinical course of 10–20 years [2]. The recent report of a Kaposi sarcoma-associated herpes virus (KSHV) in the dendritic cells of myeloma patients, whose genome contains the homologue to human IL-6, suggests that the KSHV may also play an important role in the etiology of multiple myeloma [3]. A combination of melphalan and prednisone has been the standard treatment for over 30 years [4]. Permutations of the melphalan/prednisone regimen by the addition of alkylating agents and anthracyclines have not extended the median survival [5].

Dose–response evaluation had not been addressed in myeloma until the development of the VAD regimen in the early 1980s [6]. The VAD regimen, which employs continuous infusions of standard doses of vincristine and adriamycin with frequent pulses of high-dose dexamethasone, significantly increased the rapidity and degree of response by inducing apoptosis of plasma cells. However, the median survival was not significantly different from results obtained with melphalan-based regimens [7].

Increasing the dose intensity of alkylating agents, predominantly melphalan, has unequivocally resulted in more marked and rapid tumor cytoreduction, improving the CR incidence from approximately 5% with standard melphalan–prednisone to 20%–25% with single autotransplant and 40%–50% with tandem autotransplant [8–21]. McElwain and colleagues were the first to explore high-dose intravenous melphalan (140 mg/m^2) in high-risk, newly diagnosed or advanced and refractory myeloma patients without growth factor or stem cell support [22]. The marked tumor cytoreduction observed in this small trial prompted additional studies of high-dose melphalan, initially in refractory patients but more recently in newly diagnosed patients.

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Autologous transplants

The significant morbidity and mortality associated with the prolonged marrow aplasia in the initial high-dose trials were abrogated with a series of transplant-supportive advances: the introduction of hematopoietic growth factors and peripheral blood stem cells. This combination of technologies reduced the duration of cytopenias from two weeks or longer to less than one week [23]. The transplant-related mortality was markedly reduced from 15%–35% to less than 5% [20,21]; morbidity, similarly, was decreased to the extent that high-dose therapy with autotransplant has been safely performed completely in the outpatient setting in select patients [24]. Worldwide trials in high-dose therapy for various stages of myeloma have proliferated as a result of the reduction in morbidity and mortality. Approximately 1500 autotransplants have been conducted, predominantly as part of phase II studies attempting to improve safety and efficacy (Table 1). Critical observations reported from these studies can be summarized as follows: CR rates, event-free survival (EFS), and overall survival (OS) were superior in previously untreated myeloma patients to the rates observed with historical controls treated with conventional therapy [11–13,18,21]; EFS and OS in patients with primary refractory and resistant relapse disease were also improved compared to historical controls [19,20]. In addition, this prolongation in survival was associated with improvement in quality of life and was cost-effective compared to conventional treatment [25].

The concept of tandem autotransplants was introduced by the University of Arkansas [19–21] and other groups [26,27]. The largest single-institution trial was reported by the Arkansas group: 496 patients were enrolled in the group's tandem transplant trials [20]. The authors reported an improvement in EFS and OS as dose intensity was increased from a single transplant to tandem

Table 1. Phase II studies for multiple myeloma

Regime	Tx	Reference	Prior Rx	*N*	F/U (yrs)	% ED	% CR	Median (yrs) EFS	Median (yrs) OS
Mel 200	BM	13	−	53	2.6	2	75	2	6.7
Mel-TBI	BM	14	±	52	—	2	40	2.6	4.2
BU-CY ± TBI	BM/PB	16	+	63	—	25	30	0.8	2.8
Mel ± TBI	BM/PB	17	+	207	—	4	46	2.4	2.7
Mel-TBI	PB	10	±	73	2.3	3	44	2	4.5
TBI-CC	PB	8	−	63	7.5	11	20	3.6	6.4
Mel-TBI	BM/PB	12	−	133	3	4	37	2	3.8
Mel 200 × 2	PB	32	−	231	3	2	37	3.6	5.2

Abbreviations: BM, autologous bone marrow transplant; PB, peripheral blood stem cell transplant; Mel, melphlan; TBI, total body irradiation; BU-CY, busulfan-cyclophsophamide; CC, combination chemotherapy; Tx, transplant; Rx, treatment; F/U, follow-up; ED, early death; CR, complete remission; EFS, event-free survival; OS, overall survival.

transplant, without any increase in transplant-related mortality. The cytoreductive regimen for the first transplant consisted of intravenous melphalan 200 mg/m^2. Patients with at least a sustained partial remission (PR) were offered a second cycle of identical therapy, by design within 3–6 months. Patients not achieving PR or relapsing after the first transplant were offered melphalan 140 mg/m^2 with total body irradiation (TBI; 850–1125 cGy). Those not qualifying for TBI received other combination chemotherapy. Thirty-one patients under age 55 with HLA-compatible sibling donors underwent allogeneic transplant for second transplant. Upon hematologic recovery following the second transplant, interferon (IFN) maintenance was offered to all the autotransplant patients at 3×10^6 units/m^2 thrice weekly until relapse. In the 496 patients enrolled in tandem transplant trials, analysis of disease characteristics showed that this was predominantly a heavily pretreated and high-tumor-burden group of patients, including Durie–Salmon stage III at diagnosis (48%), beta-2 microglobulin (B2M) more than 2.5 mg/L (44%), C-reactive protein (CRP) more than 4.0 mg/L (49%), refractory disease (37%), LDH more than 190 U/L (12%), and greater than 12 months of prior therapy (54%).

Clinical endpoints defined by the Arkansas group included transplant-related mortality within 60 days of first and second transplant and disease response. A PR required tumor-mass reduction of at least 75%, including reduction in Bence Jones proteinuria to less than 100 mg/day and bone marrow aspirate and biopsy with 5% or less of plasma cells. A CR was defined by absence of monoclonal protein in serum and urine by electrophoresis and immunofixation analysis, in addition to a normal bone marrow aspirate and biopsy.

Seventy-three percent (13 additional patients were awaiting second transplant at the time of the analysis) of patients completed the planned tandem transplant, with an EFS and OS of 26 and 41 months, respectively (Figure 1). Twenty-three percent of the 470 treated patients achieved CR after the first transplant, and 42% of 321 patients were in CR after the second transplant. CR rates were higher in patients with chemotherapy-sensitive disease ($p = 0.0001$), IgA isotype ($p = 0.005$), preserved albumin (≥ 3.5 mg/dL; $p = 0.007$), and less than 12 months of prior therapy ($p = 0.03$). Patients with primary refractory disease and resistant relapse had similar CR rates of 11% and 14%, respectively. Transplant-related mortality was 1% after the first transplant and 4% after the second transplant. Twelve percent of patients died within 12 months of first transplant, 7% from treatment-related complications and 5% due to disease progression. A recent update expanded to 542 patients demonstrated a projected five-year EFS and OS of 30% and 45%, respectively [28].

Multivariate regression analysis of pretransplant prognostic features demonstrated that the absence of unfavorable karyotypes (any translocation and abnormalities of chromosomes 11 and 13), low B2M and CRP, less than 12 months of prior therapy, and chemotherapy-sensitive disease were all inde-

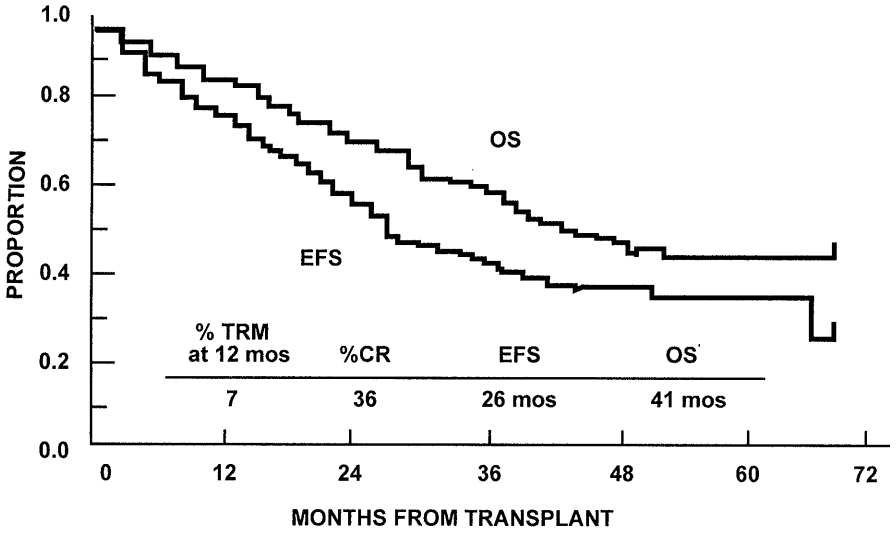

Figure 1. EFS and OS from the first transplant. TRM within 12 months of first transplant was 7%; 36% of patients achieved CR. Median durations of EFS and OS were 26 and 41 months, respectively.

Table 2. Multivariate analysis of pretreatment prognostic variables

Favorable	EFS	Favorable	OS
<12 mos. prior Rx	0.0001	No 11/13	0.0001
No 11/13	0.0001	B2M <2.5 mg/dL	0.0001
B2M <2.5 mg/dL	0.0001	Sensitive	0.0007
CRP <0.4 mg/dL	0.002	CRP <0.4 mg/dL	0.002
Sensitive	0.01	<12 mos. Prior Rx	0.02
Non-IgA	0.02	LDH >190 U/L	0.06

Abbreviations: No 11/13, no translocations or abnormalities of chromosomes 11 and 13.

pendently favorable prognostic indicators for EFS and OS (Table 2). Non-IgA isotype was a favorable feature for EFS but not for OS. Four distinct risk groups could be identified (through the use of cytogenetics, B2M, and duration of prior therapy), thus identifying high-risk patients who would be candidates for novel and innovative new therapies (Table 3).

In order to evaluate the prognostic implications of CR posttransplant and of timely administration of two transplants within six months, a multivariate regression landmark analysis was performed that was restricted to the 380 patients who survived at least six months after the first transplant, corresponding to the time interval originally intended for completion of a second transplant (Table 4). The landmark analysis identified the absence of abnormalities

Table 3. Clinical outcome according to favorable pretransplant prognostic variables

No. variables[a]	*N*	EFS	OS
3	147	39	72+
2	241	28	48
1	125	16	24
0	29	8	11

[a]No abnormalities of chromosomes 11 and 13, low B2M, <12 months prior therapy.

Table 4. Multivariate analysis of posttransplant prognostic variables: six-month landmark

Favorable	EFS	Favorable	OS
<12 Mos. Prior Rx	0.0001	No 11/13	0.0001
No 11/13	0.0001	B2M <2.5 mg/dL	0.0002
B2M <2.5 mg/dL	0.0001	CR	0.001
Non-IgA	0.002	CRP <0.4 mg/dL	0.003
CR	0.002	<12 Mos. Prior Rx	0.009
CRP <0.4 mg/dL	0.004	Non-IgA	0.01

Abbreviations: no 11/13, no translocations or abnormalities of chromosomes 11 and 13.

of chromosomes 11 and 13, attainment of CR, less than 12 months of prior therapy, low B2M and CRP, and non-IgA isotype as independent favorable variables for EFS and OS. Timely administration of a second transplant and CR induction within six months benefited especially the poor-risk group (< two favorable features). Good-risk patients (≥ two favorable features) enjoyed superior EFS, although the extension of OS did not reach statistical significance (Table 5).

In contrast to other reports that myeloablative therapy is of limited value for late myeloma [29] and does not benefit patients with sensitive disease early in their disease course [30], the Arkansas results demonstrated that a large majority of myeloma patients benefited from this approach. Fifty-four percent of patients received standard therapy in excess of 12 months; the CR rate was 28%, with a median EFS and OS of 21 and 42 months, respectively, exceeding the results reported in historical controls treated with conventional chemotherapy. Compared to resistant relapse, sensitivity to standard therapy conferred a significantly higher CR rate (46% vs. 14%) and effected prolongation of both EFS (31 vs. 20 months) and OS (48 vs. 30 months) (all $p = 0.0001$). Patients with primary refractory disease had much lower CR rates (11%; $p = 0.0001$) and inferior OS ($p = 0.05$) than those with sensitive disease. These patients, however, fared significantly better than patients with refractory relapse (Table 6).

Table 5. Contribution of timely second transplant and complete remission in good- and poor-risk groups (B2M <2.5, CRP <0.4, with either non-IgA or no 11/13): six-month landmark

			Median months			
	N		EFS		OS	
Risk	Non-IgA	No 11/13	Non-IgA	No 11/13	Non-IgA	No 11/13
Good[a] 2Tx[b] or CR	221	228	39	39	66+	66+
No 2Tx and No CR	86	81	23	23	47	48
	p		0.0001	0.0001	0.006	0.008
Poor[a] 2Tx or CR	90	83	25	22	37	35
No 2Tx and No CR	43	48	12	11	18	18
	p		0.0008	0.002	0.0001	0.002

[a] Good ≥2, poor <2 favorable pretransplant variables.
[b] Timely second transplant (within six months after first transplant).

Table 6. Effect of pretransplant disease status on transplant outcome

Parameter	*N*	% CR	EFS	OS	*p*
Sensitive	292	36	29	52	0.05
1° Refractory	109	11	23	39	0.008
Resistant relapse	69	14	14	25	0.002

In summary, tandem transplants are safe and feasible, with over 80% of patients expected to complete the planned two cycles of high-dose therapy within one year. CR was achieved in 36% of patients, with a transplant related mortality of 7% within the first year. EFS and OS were markedly extended, regardless of pretransplant risk features (defined by B2M and CRP levels), when CR was attained within six months posttransplant, when the first transplant was conducted within 12 months of initiating standard therapy, and when the second transplant was applied within six months from the first. Cytogenetic abnormalities (translocations with 11q and −13/−13q) were recognized as a dominant unfavorable prognostic factor [31]. The critical importance of low B2M and CRP for favorable outcome has been appreciated with both standard therapies and also with autotransplants.

Autologous transplants in newly diagnosed patients

The superiority of these results by the Arkansas group and others over those observed after conventional chemotherapy is difficult to assess because patient selection for autotransplant is subject to considerable bias, including young age, good performance status, normal renal function, physician preference,

and institutional referral patterns. Prospective randomized trials were required to compare conventional chemotherapy with high-dose therapy. This need led to the French Myeloma Intergroup (IFM) trial, which consisted of four months of VMCP alternating with VBAP chemotherapy followed by randomization to autotransplant using melphalan 140 mg/m^2 plus low-dose TBI (800 cGy) versus eight additional months of VMCP/VBAP [18]. They observed superior CR rates (22% vs. 5%, $p \leq 0.001$), EFS (28% vs. 10%, $p = 0.01$), and OS at five years (52% vs. 12%, $p = 0.03$), respectively, for the high-dose arm compared to the conventional arm.

The largest autologous trial in newly diagnosed patients has been reported by the University of Arkansas [32]. The 'Total Therapy' regimen consisted of remission induction with mutually non-cross-chemotherapy followed by double transplants (VAD $\times$ 3, high-dose cyclophosphamide with GM-CSF for stem cell mobilization, EDAP, and tandem transplants using melphalan 200 mg/m^2). Upon completion of the tandem transplant, patients received maintenance IFN at 3 $\times$ 10^6 mu/m^2 thrice weekly until relapse. Patients were eligible up to age 70 and had received at most one cycle of previous chemotherapy. Of the 231 patients enrolled in this study, 84% completed one transplant and 71% completed tandem transplant. Of the 164 patients who completed tandem transplants, the PR and CR rates were 94% and 48%, respectively. With an intent-to-treat analysis for all patients, these rates were 81% and 38%. The median EFS and OS were 43 and 62 months, respectively.

A pair-mate analysis of 116 previously untreated patients on the Total Therapy trial were matched for age, B2M, and renal function with 116 patients receiving standard therapy, mainly VAD, as part of Southwest Oncology Group (SWOG) trials [21]. Total Therapy induced higher response rates (PR 85%; CR 40%), with an EFS and OS of 49 and 62+ months, respectively (median follow-up 31 months). This outcome compared to the response rates (PR 52%; CR not available) and the EFS and OS of 22 and 48 months, respectively, for the SWOG standard therapy group. Thus, as in the IFM randomized trial, this pair-mate analysis revealed superior remission rates and prolonged EFS ($p = 0.0001$) and OS ($p = 0.01$) duration among the transplanted patients (intent-to-treat). Results of the standard SWOG treatment were superior to the IFM standard treatment arm, possibly due to patient selection differences. The CR rate of 40% with tandem transplants was almost twice the IFM rate of 22%; the median EFS of 49 months with Total Therapy exceeded the 27-month median of the IFM trial after a single transplant, supporting the concept that greater tumor cytoreduction was effected by a tandem versus a single transplant (Table 7). This improvement in EFS reported by the Arkansas group has prompted the current IFM randomized trial comparing single versus tandem transplant: preliminary analysis shows comparable two-year survival between the single and tandem transplant groups [33]. Additional follow-up is required to confirm these preliminary results.

Talbe 7. Comparative trials of standard versus high-dose therapy

	French ≤65 years *Randomized* [18]		Arkansas ≤70 years *Pair Mate* [21]	
	Standard	High-dose	Standard	High-dose
N	100	100	116	116
%CR	5	22	NA	40
EFS mos.	18	27	22	49
OS mos.	37	60+	48	62+

Timing of transplantation

The next major question is to determine the optimal timing of myeloablative therapy: early following remission induction or as salvage therapy after conventional therapy fails. Preliminary results from another French Myeloma Intergroup randomized trial comparing early versus delayed transplant demonstrated comparable survival in both groups [34]. Investigators in the United States and Canada are currently participating in a randomized North American Intergroup Trial (INT 141) that compares high-dose melphalan 140 mg/m^2 plus TBI 1200 cGy with PBSC support with VBMCP standard chemotherapy. The mandatory PBSC collection on the standard treatment arm and the plan for salvage transplantation upon relapse on VBMCP assures that all patients are candidates for myeloablative therapy, an important ethical issue in light of improved results with high-dose therapy suggested by several phase II autotransplant trials, the IFM randomized trial, and the Arkansas pair-mate analysis. Thus, INT 141 will address the question of optimal timing of myeloablative therapy, similar to the current French trial.

Transplants in high-risk patients

Due to their frequently compromised renal function and advanced age, many patients with multiple myeloma are often not considered for high-dose intensive therapy. The Arkansas group has successfully performed high-dose therapy trials in these 'high-risk' patients. Based upon the recent demonstration that melphalan pharmacology is not affected by renal function [35], they reported the outcome of 23 patients with renal insufficiency (creatinine >2 mg/dL), including seven patients on chronic hemodialysis, treated with high-dose melphalan 200 mg/m^2 and autotransplant compared to 46 patients with normal renal function who were matched for CRP, immunoglobulin isotype, duration of prior therapy, and chemotherapy sensitivity [36]. No significance differences in hematologic recovery, toxicities, early death, or CR were observed. Although the EFS figures were comparable, patients with normal renal function demonstrated a trend for superior OS (30 months vs. 16 months, p = 0.09). Furthermore, improvement in renal function (creatinine ≤1.2 mg/dL)

was observed in 43% of the 23 patients with renal insufficiency following autotransplant. Ballester et al. have reported similar findings in a smaller group ($n = 6$) of patients [37].

Analysis of the Arkansas data for patients 60 years and older showed comparable pretransplant prognostic features to patients under 60 [38]. Age of 60 years or greater was an adverse feature for transplant-related mortality (6% vs. 1%, $p = 0.001$) and CR (23% vs. 56%, $p = 0.01$). However, in a multivariate regression analysis, age was not an unfavorable prognostic factor for EFS or OS. Similarly, in the IFM trial, age of 60 years or greater was not a significant prognostic factor [19]. The Arkansas group has performed high-dose therapy with autotransplants in 23 patients over the age of 70 (median 72 years), including one at age 82 [39]. Complete remission was achieved in 17%, with an EFS and OS of 9 and 23 months from transplant, respectively; transplant-related mortality was 9%. In addition, hematopoietic stem cell reserve is maintained regardless of age: stem cell mobilization and engraftment kinetics were similar in younger and older patients [40]. In summary, high-dose therapy should not be withheld from the majority of patients based solely upon advanced age and renal failure who have the most to gain from effective tumor cytoreduction.

Relapse after transplant

An important question posed by patients and referring physicians relates to the management of myeloma patients who relapse following high-dose therapy. This issue is especially important to younger patients and those who undergo myeloablative therapy early in their disease course. The Arkansas group previously reported their experience in patients with recurrent disease after transplant [41]. A recent update included a total of 196 patients: 105 relapsed after a single transplant and 91 after tandem transplants [42]. Of these patients, 49 proceeded directly to second transplant; 147 received standard therapy as initial therapy. Overall, posttransplant EFS and OS were 8 and 14 months, respectively. In those patients who received a second transplant as primary salvage therapy, especially in the presence of a low B2M, the EFS and OS were 64+ and 64+ months, respectively. For patients who relapsed after tandem transplant, late relapse (>12 months) and low B2M predicted for an EFS and OS of 8 and 15 months, respectively, compared to 5 and 9 months, respectively, for those with early relapse and high B2M.

Risk of myelodysplasia posttransplant

One of the major concerns in long-term myeloma survivors is the potential development of myelodysplastic syndrome (MDS) and secondary acute myelogenous leukemia (AML), which has been reported both for Hodgkin's disease [43] and non-Hodgkin's lymphoma [43,44] following high-dose therapy and autotransplant. This finding is highly relevant, since most stan-

dard therapies consist of protracted courses of alkylating agents, especially melphalan and nitrosoureas. In addition, high-dose melphalan with or without TBI is the most widely used preparative regimen for high-dose therapy. The Arkansas group examined 71 patients enrolled on the Total Therapy program, which limits alkylating agent use (< one cycle of standard therapy and virtually no stem cell toxic induction therapy prior to PBSC collection) to 117 patients who had more than one year of conventional chemotherapy [45]. They observed 0 versus 7 cases of MDS, respectively, in the two groups, for a projected incidence of 12% at 48 months. This finding implies that prolonged standard-dose alkylating-agent use, rather than myeloablative conditioning chemotherapy, is primarily responsible for secondary MDS/AML in this patient population. Thus, these results suggest that avoidance of stem-cell-damaging alkylating agents prior to PBSC collection is essential to prevent future secondary hematologic malignancies.

Hematopoietic stem cells

Prompt hematopoietic reconstitution is necessary to rescue patients from high-dose therapy. Autologous peripheral blood stem cell (PBSC) transplant has virtually supplanted autologous bone marrow transplant due the more rapid engraftment kinetics. Several investigators have reported that the number of CD34+ cells infused is an important predictor of hematologic recovery after PBSC infusion: an optimal dose of 5 ± 10^6 CD34+ cells/kg results in rapid and complete engraftment [46,47]. Lower cell doses, however, can produce successful grafts, with a proportion of patients having slower recovery of platelets. PBSC mobilization can be accomplished with high-dose hematopoietic growth factors, chemotherapy alone, or a combination of growth factors plus chemotherapy. The duration of prior exposure to chemotherapy was an important predictor of CD34+ cell yield following mobilization with cyclophosphamide and GM-CSF [47]. Although chemotherapy with hematopoietic growth factor support produces the highest yield of CD34+ cells, it is often associated with significant toxicity with limited tumor cytoreductive effect. In contrast, high-dose granulocyte colony-stimulating factor (G-CSF; 10–16 μg/kg) yields lower quantities of CD34+ cells but is well tolerated with minimal morbidity. The Arkansas group has recently completed a randomized trial comparing high-dose G-CSF to high-dose cyclophosphamide plus G-CSF. As expected, they observed a lower number of CD34+ cells in the G-CSF alone group (5 vs. 49×10^6 CD34+ cells/kg, $p = 0.03$); however, the posttransplant engraftment kinetics were comparable to those of the high-dose cyclophosphamide plus G-CSF group [48]. The Seattle group has reported similar results in a nonrandomized trial of cyclophosphamide/etoposide plus G-CSF vs. cyclophosphamide plus G-CSF vs. G-CSF alone [46]. They also found that marrow disease, prior radiation therapy, number of cycles of prior chemotherapy, and patient age were important pretreatment factors determining the CD34+ cell yield [49].

Stem cell selection and tumor purging

One of the most difficult issues in autotransplantation in multiple myeloma is tumor cell contamination in the autograft. There are substantial data that myeloma and premyeloma cells are present in the circulating blood. PBSCs mobilized with either high-dose chemotherapy and/or hematopoietic growth factors are contaminated with clonal plasma cells as determined by morphology, immunoglobulin gene fingerprinting, and CDR 3 analysis of the immunoglobulin hypervariable region. The percentage of circulating clonal plasma cells has varied between less than 0.01% and more than 10%, although most patients were between 0.1 and 1.0% [50]. Autograft decontamination of tumor cells has been approached by either positive selection or negative selection by monoclonal antibodies and/or cytotoxic agents [14,51–53].

The most stringent positive selection technique reported utilizes high-speed sorting for CD34+Thy+Lin- phenotype, which results in a 2.7–7.3 log depletion of tumor cells [54]. Tumor cytoreduction with tandem transplants has resulted in molecular remissions [27,55]. Unfortunately, early relapses have been noted even in minimally pretreated patients. Furthermore, although trilineage hematopoiesis was successfully demonstrated, this was delayed, especially for platelet recovery, and was also associated with profound immunosuppression when compared with nonselected PBSC. A nearly completed multi-institutional randomized trial comparing CD34+ selected (biotin/avidin column technology) to nonselected cells will help guide clinical investigators into the next generation of selection approaches.

Allogeneic transplants

Autologous stem cell transplants have wide applicability in the treatment of patients with multiple myeloma. Autologous transplantation has been shown to be superior to conventional chemotherapy in a controlled randomized trial [18]. Thus, the trend has moved towards autotransplant early in the disease course as part of front-line treatment. Nevertheless, current approaches to autotransplantation are probably not curative, even in the best prognostic groups [20,56]. Although long-term survival after autotransplant has been described, a plateau in survival has not been observed in any of the published trials, implying that few, if any, of these patients are cured. The etiology of disease relapse is unknown: is relapse due to persistent residual disease from inadequate myeloablative therapy and/or from reinfusion of tumor-containing autografts? Further intensification of preparative regimens will probably not significantly improve current high-dose regimens due to the risk of increased extramedullary toxicities. Even with newer techniques to deplete many logs of tumor cells from the autografts [54], it is unlikely that the majority of myeloma patients will be cured with autotransplantation. Posttransplant immunomo-

dulation, discussed below, is an area of intense investigation to improve survival by eradicating minimal residual disease.

Allogeneic transplants, in contrast, may be curative, since they have the advantage of tumor-free grafts and the putative existence of a graft-versus-myeloma effect. Because the median age of myeloma patients is 65 years, less than 10% of patients enrolled into cooperative trials in the United States are candidates for matched sibling donor transplants. This estimate considers an age limit of 55 years and a matched sibling donor availability in 25% of patients.

Over 500 allotransplants for multiple myeloma have been performed worldwide [57]; the largest trials have been reported from the European Bone Marrow Transplant Registry (EBMTR) [58], International Bone Marrow Transplant Registry (IBMTR) [59] and single-institutional trials from Seattle [60], Arkansas [61], the Dana-Farber Cancer Institute [14], and Vancouver [62]. Results of the these reports are summarized in Table 8. It should be noted that considerable overlap exists from the two registries (EBMTR and IBMTR), complicating the separate interpretation of their results. The median ages for patients in these studies range from 42 to 48; the percentage of patients with chemotherapy-responsive disease ranged from 21% to 100%, with complete remissions achieved in 22% to 58% of patients. The EBMTR data are the most mature: relapse-free survival at six years for the patients achieving CR was 34% (although this is only 9 of the 162 patients), and actuarial survival for all patients at seven years was 28% in a predominantly chemosensitive, minimally treated patient population. This outcome compares to a relapse-free survival of 33% at five years for the patients achieving CR, with an overall survival of 20% and progression-free survival of 16% in a predominantly chemoresistant, heavily pretreated patient population in the Seattle series. This result is quite similar to the Arkansas data in a comparable group of high-risk patients (heavily pretreated, chemotherapy resistant). Overall, approximately 40% of all patients receiving allogeneic transplants achieve a CR, with 30% to 50% of those achieving CR remaining disease-free 3 to 6 years after transplant. Unfortunately, there is an exceedingly high

Table 8. Allogeneic transplants for multiple myeloma

Source	No. patients	Median age (range)	Responsive disease (%)	TRM deaths (%)	CR post-Tx (%)
IBMTR [59]	257	42 (22–59)	42	50	44
EBMTR [58]	189	43 (23–59)	76	41	48
Seattle [60]	80	44 (28–58)	21	56	35
Arkansas [61]	59	45 (32–54)	34	24	34
DFCI [14]	21	43 (37–55)	100	9	33
Vancouver [62]	19	48 (28–54)	80	16	58

Abbreviations: TRM, transplant-related mortality; CR, complete remission; Tx, transplant; DFCI, Dana-Farber Cancer Institute.

transplant-related mortality, approaching 50% at one year posttransplant, and late relapses still occur as long as seven years posttransplant.

Prognostic features in allogeneic transplants

Due to the considerable heterogeneity in patient demographics and prior treatment history, it is difficult to predict outcomes of allogeneic transplant from the available published trials. The EBMTR analysis showed that stage I disease at diagnosis, only one prior chemotherapy regimen, female gender, and pretransplant CR were associated with a favorable outcome; improved survival was observed in patients with low B2M and female gender, who received only one line of treatment prior to transplantation [58]. The IBMTR analysis showed good performance status (≥80%) and chemotherapy sensitivity associated with the probability of survival [59]. Evaluation by the Seattle group of prognostic features for early mortality revealed that patients transplanted more than one year from diagnosis and Durie–Salmon stage 3 disease had a 1.8 and 2.0 greater risk, respectively, of dying from any cause. The risk of relapse or disease progression was greater for female recipients receiving allografts from male donors and for patients who had received more than eight cycles of prior chemotherapy (3.5 and 4.3 times greater risk, respectively). Only Durie–Salmon stage 3 patients at the time of transplant influenced transplant-related mortality/relapse/progression-free survival (1.9 times greater risk than Durie–Salmon stage I/II) [60]. Finally, the Arkansas analysis found that patients with chemotherapy-sensitive disease and maintained albumin (>30.5 g/dL) had a superior outcome with allogeneic transplant [61].

In analyzing these prognostic variables, it appears that patients with less extensive prior therapy, low disease burden (as determined by B2M), female gender, chemotherapy-sensitive disease, good performance, and nutritional status would be predicted to have the most favorable outcome. However, it is this same group of patients who would be projected to have a superior outcome with conventional chemotherapy with or without autotransplant.

Alternate donors

The number of transplants performed from alternate donors is quite limited and has usually been reserved for patients with advanced, refractory myeloma. In total, 39 patients have been reported from the Seattle program, the European Bone Marrow Transplant Registry, and the Vancouver groups [57]; the majority were matched unrelated transplants. Although there have been occasional long-term survivors (8 of 39; range 1.5 to 8.5 years), the majority expired from treatment-related toxicities. Although the use of alternate donors for allogeneic transplant is technically feasible, further studies are necessary to determine the safety and efficacy of this approach.

Graft-versus-myeloma effect

A graft-versus-leukemia effect has been strongly suggested following allogeneic transplantation: relapse rates in leukemic patients receiving syngeneic and T-cell-depleted transplants occur more frequently [63], and allogeneic recipients who do not develop graft-versus-host disease (GVHD) relapse more frequently than recipients who do develop GVHD [64]. Recently, the utilization of donor leukocyte infusions (DLI) in disease relapse after allogeneic transplant has resulted in durable complete remissions in 50% to 80% of patients with chronic myelogenous leukemia but more sporadically in patients with other relapsed hematologic malignancies [65]. A graft-versus-myeloma effect (GVM) has been reported in small numbers of patients with persistent disease or relapse after allogeneic transplant either by rapid immunosuppressant withdrawal [66] or by DLI [67–71]. In the five published cases of DLI, three have achieved CR and two PR. The number of cells infused, duration of response and severity of associated GVHD varied considerably in these reports. The largest experience has been reported by the Netherlands group [71]. They observed responses in 8 of 14 patients, included four CRs, after DLI for relapse after allogeneic transplant. This response was cell dose dependent and associated with the development of chronic GVHD. The median response duration was five months. However, only 3 of the 8 responders were in continued CR at 7, 18, and 36 months postinfusion. These anecdotal case reports support the principle of a GVM effect. To further establish the efficacy and the immunologic milieu of the GVM effect, the Eastern Cooperative Oncology Group (ECOG) will initiate a multi-institution trial of DLI in multiple myeloma patients with persistent or relapsed disease following allogeneic transplant. Finally, since the GVM effect may also be associated with significant GVHD, a few centers have initiated trials of herpes simplex virus–thymidine kinase (HSV-TK) transduced donor lymphocytes as the source of DLI [72]. Patients who develop severe GVHD will receive gancyclovir to eliminate the donor lymphocytes, thus abrogating the GVHD.

Autologous versus allogeneic transplatation (Table 9)

Due to the exceedingly high transplant-related mortality with allogeneic transplants, which has been consistently reported in the 50% range within the first year, it is critical to determine whether allogeneic transplants effect an outcome superior to autologous transplants. The EBMTR recently compared a retrospective matched case–control analysis of 378 patients, 189 with each modality [73]. The patients were matched for the most important prognostic features, namely, gender and number of prior chemotherapy regimens before transplantation. The groups were comparable with the exception that the median age for allogeneic transplant was 43 years compared to 49 years for autotransplant and the median posttransplant follow-up was of shorter dura-

Table 9. Autologous versus allogeneic transplant

Group	*N*	Autologous		Allogeneic	
		TRM (%)	OS (mos.)	TRM (%)	OS (mos.)
EBMTR [73]	189	13	34	41	18
Arkansas [74]	63	2	48	25	10
Toronto [75]	22	5	>48	27	7

Abbreviations: TRM, transplant-related mortality; OS, overall survival.

tion in the autotransplant group (46 months for allogeneic transplant versus 30 months for autotransplant). The complete remission rate was similar in both groups. The overall survival with autotransplant was superior to that with allotransplant, with a median survival of 34 months and 18 months, respectively (Table 9). The predominant difference for the poorer survival in the allotransplant group was the higher treatment-related mortality of 41% versus 13% for the autotransplant group which was not compensated for by lower rate of relapse and progression. However, for patients alive at one year posttransplant, there was a trend for improved overall survival ($p = 0.09$) and significantly better progression-free survival ($p = 0.02$). Interestingly, as reported in the previous EBMTR analysis of prognostic features in allotransplantation, in which female gender had a superior outcome, an analysis of overall survival for women and men showed that the survival difference was significant only in men owing to the better survival in women, due to lower transplant-related mortality in women following allotransplantation. The reason for the female survival advantage after allotransplant is unclear. A review of the allotransplant registry data revealed that the allotransplant-related mortality in multiple myeloma showed no tendency to improve with time, in contrast to the 20% reduction after 1988 in chronic-phase chronic myelogeneous leukemia. Similar observations have been reported by the Seattle group [57]. A pair-mate analysis was also conducted by the Arkansas group in 63 allotransplant recipients [74]. The autotransplant recipients demonstrated a superior event-free and overall survival duration of 27 and 48 months, respectively, compared to 8 and 10 months, respectively, among allotransplant recipients. This outcome was mainly due to a considerably higher transplant-related mortality of 25% with allotransplant compared to 2% with autotransplants in the first 60 days. A third comparison has been reported by the University of Toronto transplant program in a consecutive series of myeloma patients who underwent autologous ($n = 40$), allogeneic ($n = 22$), or syngeneic ($n = 2$) transplantation [75]. They observed a 5% versus 27% transplant-related mortality at 90 days in the autologous and allogeneic transplant groups, respectively. With a median follow-up of 15 months in the autotransplant group and 42 months in the allotransplant group, the three-year progression-free survival was 17% (autologous), and the 22% (alloge-

neic), and the three-year overall survival was 74% (autologous) and 32% (allogeneic). They conclude that low early mortality and high overall survival of autotransplantation compares favorably with both the results of allotransplantation and standard therapy. Finally, the National Cancer Institute High Priority Multiple Myeloma Intergroup Trial 141, which allowed allotransplants in patients up to age 55 with an HLA-identical sibling donor, utilizing the identical myeloablative regimen in the autotransplant arm, has been temporarily closed after an interim analysis noted a 41% transplant-related mortality within the first 60 days (less than 5% in the autotransplant arm).

Posttransplant immunomodulation

Interferon-alpha has been variably reported as beneficial or lacking clinical efficacy in myeloma trials for maintenance therapy as well as when utilized together with combination alkylator therapy for remission induction [76,77]. IFN was used posttransplant in the randomized IFM and Arkansas trials, based upon promising results of previous pilot data from France and England [78,79]. The randomized Royal Marsden trial reported superior EFS and OS in the cohort of patients treated with IFN, observed only in patients with a CR following autotransplant. Despite the use of IFN in the French [18] and Arkansas trials [80], no plateau has been observed in the EFS curve in autotransplant. A pilot trial of IFN dose-escalation postallogeneic transplant by the EBMTR was discontinued due to the development of a toxic death due to acute GVHD at the second dose level (2 million units thrice weekly) and significant GVHD in 4 of 5 recipients at the third dose level (3 million units thrice weekly) [81]. The INT 141 trial also has a randomization to IFN or observation following transplant or completion of standard therapy.

New strategies are required to control minimal residual disease post-transplant to reduce the risk of relapse (Table 10). Maintenance chemotherapy with regimens found to be highly effective in the setting of salvage therapy after autotransplant, such as cyclophosphamide, dexamethasone, etoposide, and

Table 10. Maintenance therapy in multiple myeloma

Conventional	High-dose
Interferon	Interferon
Biological response modifiers	Autologous GVM
Vaccines	Vaccines
Soluble idiotype	Soluble idiotype
Dendritic cell pulsing	Dendritic cell pulsing
Interleukins	Monoclonal antibodies
	Interleukins
	Activated NK cells
	Chemotherapy (CDEP)

cisplatin (CDEP), has been reported by the Arkansas group [82]. Alternate approaches that utilize immunologic manipulations such as idiotype or dendritic cell vaccination, interleukins, and B-cell directed immunotoxin are being investigated [83–86]. The exploitation of a graft-versus-myeloma effect in both the allogeneic and autologous [87] setting is provocative. Other approaches may focus on the generation of idiotype-specific allogeneic cytotoxic T lymphocytes to avoid GVHD and on means to increase the immunogenicity of tumor cells by transduction with granulocyte-macrophage colony-stimulating factor or other cytokines [88].

Summary and future perspectives

The clinical trial results of high-dose therapy with autotransplantation in multiple myeloma permit the following conclusions: high-dose therapy with autotransplants is superior to conventional therapy in previously untreated patients; true CR can be achieved in approximately 20%–25% of newly diagnosed patients with single transplant and 40%–45% with tandem transplants; myeloablative therapy can be safely administered in patients up to and exceeding 70 years of age and those with renal insufficiency. Although CR rates have improved with high-dose therapy, plateaus are not yet apparent for either EFS or OS after autologous transplant, whereas plateaus do appear to be the case in small numbers of patients after allogeneic transplants. Recent experience with autotransplant trials in myeloma attests to the role of autotransplants in the palliative setting, with attainment of a high quality of life and a procedure-related mortality of less than 5%. The observation by Jagannath et al. that approximately 10% of patients with advanced and refractory myeloma maintained a CR almost 10 years after autotransplant suggests that a cure may be achievable, especially when tandem transplants are performed early in the disease course [89]. This suggestion is further supported by the observations that molecular remissions have been observed following tandem autotransplant [27,55].

An important lesson from standard chemotherapy trials in myeloma over the past three decades is that standard melphalan and prednisone or similar approaches should be avoided, especially since the prolonged administration of melphalan and nitrosoureas decreases the likelihood of successful stem cell procurement while increasing the risk for secondary MDS or AML, which may be aggravated by subsequent high-dose therapy with autotransplants. Initial treatment with VAD-like regimens or pulse dexamethasone and PBSC collection of sufficient quantity to support two future transplants is strongly recommended in all patients. The recently completed randomized IFM trial comparing one autotransplant with two autotransplants should provide essential information about the effectiveness of sequential cytoreductive high-dose therapies. The INT 141 trial should answer the question of whether optimal timing of autotransplant is as consolidation of induction therapy or as salvage

therapy at the time of relapse. Numerous ongoing studies are aimed at improving stem cell mobilization and decontamination of tumor cells in autografts. Eradication of minimal residual disease posttransplant is the focus of intense clinical investigation.

With regard to allogeneic transplantation, the high transplant-related mortality in multiple myeloma must be reversed in order to offer this potentially curative option to eligible patients. Methods to decrease transplant-related mortality will concentrate on optimizing host-, disease-, and treatment-related features by the following means: limiting transplants to patients with good performance and nutritional status and to less heavily pretreated patients with lower tumor burden and chemotherapy-sensitive disease; improving conditioning regimens with less intensive preparative regimens (*minitransplants*); and depleting T cells to allow for posttransplant immunotherapy, with donor lymphocyte infusions for patients with persistent or relapsed disease to induce a graft-versus-myeloma effect.

Finally, the recent discovery of the Kaposi sarcoma-associated herpes virus (KSHV) in the dendritic cells of patients with multiple myeloma may have substantial effects on the future management of this disease. The presence of the virus in circulating dendritic cells coupled with its absence in CD34 progenitor cells may require CD34 selection for all autografts. Preliminary reports indicate that CD34 selection is possible with current techniques [3]. Posttransplant therapy, for autologous and allogeneic transplants, may be directed toward antiviral therapy to eradicate residual KSHV in the host dendritic cells.

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8. Hematopoietic growth factors and acute leukemia

Jacob M. Rowe and Jane L. Liesveld

The hematopoietic growth factors are glycoproteins that can be produced by recombinant DNA technology. They have many potential clinical uses in acute leukemia, and these will be discussed in this chapter. Several areas have been explored extensively, and many data are available from clinical trials. Other areas are of potential interest but have a paucity of clinical information. The past decade has seen major strides in the development and clinical application of cytokines in acute leukemia, and it is expected that this trend will continue over the next decade as further areas are explored and results of clinical trials mature to enable us to determine the precise role of cytokines in the clinical setting.

Over the past decade, the development of in vitro marrow clonogenic culture systems has led to the discovery of a family of interacting hematopoietic growth factors that have recently been demonstrated to have critical physiologic roles for controlling hematopoeisis in vivo. Of the many hematopoietic growth factors that have been cloned, only granulocyte colony stimulating factor (G-CSF) and granulocyte–macrophage colony stimulating factor (GM-CSF) have seen widespread clinical use. Some trials have begun with interleukin 3 (IL-3), macrophage CSF (M-CSF), stem cell factor (SCF), IL-6, IL-1 and thrombopoietin. GM-CSF and IL-3 have predominantly proliferative effects on early hematopoietic cell compartments, whereas G-CSF and M-CSF, or CSF-1, have major differentiation as well as proliferative effects on lineage-specific cells.

There are many potential applications of colony stimulating factors in the treatment of acute leukemia (Table 1). These include reducing the period of neutropenia, inducing leukemic cells into the S phase of the cell cycle, enhancing antimicrobial function, protecting stem cells, inducing differentiation of leukemic cells, interrupting autocrine–paracrine loops, and directly inhibiting leukemic cell proliferation. This chapter will focus mainly on the first three potential uses but will also briefly touch on the other areas.

In this chapter, the data that will be considered will relate mostly to acute myelogenous leukemia (AML), where historical controversy abounds, owing to the effect of cytokines on the leukemia progenitor cells. The data for acute lymphocytic leukemia (ALL) are more appropriately considered with the

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Table 1. Potential uses of cytokines in AML

1. Reducing period of neutropenia
2. Enhancement of antimicrobial function
3. Recruitment of cells into S phase of the cell cycle (priming)
4. Induction of differentiation of leukemic cells
5. Direct antileukemic effects
6. Interruption of autocrine–paracrine loops
7. Stem cell protection

lymphoproliferative disorders. In fact, several clinical trials have been reported in ALL, mostly encouraging the use of cytokines, especially in older adults [1–3]. Nonetheless, several aspects described in this chapter for AML are probably equally applicable to ALL, especially the emerging data on the direct antimicrobial action of cytokines.

Effects of growth factors on leukemic cell proliferation

Some AML blasts possess receptors for the clinically available growth factors, G-CSF and GM-CSF [4]. G-CSF is produced by fibroblasts, monocytes, and endothelial cells. AML blasts may possess G-CSF receptors and may demonstrate enhanced proliferation in the presence of G-CSF in vitro. Autocrine production of G-CSF by AML cells has also been demonstrated. G-CSF has been found to have a protective effect on leukemic cell programmed death [5]. The role of G-CSF in the biology of AML is probably a complex one. There is evidence that G-CSF induces tyrosine phosphorylation of p95vav in primary human acute myelogenous leukemic cells but not in neutrophils [6]. Precise signaling pathways for G-CSF in primary AML blasts have not yet been completely described.

Faussat-Suberville et al. [7] have found that 50 ng/mL GM-CSF increased the leukemic cell proliferation in 9 of 16 patients, resulted in functional differentiation in four patients as measured by nitroblue tetrazolium (NBT) reduction, and enhanced plating capacity of leukemic progenitors in six cases. Most of the cases demonstrating response to GM-CSF were those that demonstrated spontaneous increased ^{3}H-thymidine uptake in culture, although some 'nonproliferative' cases also responded to GM-CSF.

AML blasts have also been found to possess receptors for many other hematopoietic growth factors (Table 2). Interleukin 11 (IL-11) is a pleiotropic cytokine. By itself, IL-11 has been found to be ineffective in supporting myeloid leukemic cell growth, but it can enhance growth stimulated by IL-3, GM-CSF, or *c-kit* ligand. Thymidine suicide studies in AML patient samples showed that 40% to 50% of the blast cells were in S phase when exposed for 16 hours to IL-3. This increased to 70% to 90% in response to either IL-11 or IL-6. Both *kit* ligand and IL-11 have also been found to support the clonogenic growth of AML blasts [8].

Table 2. Growth factors with direct in vitro action on AML cells

1. GM-CSF
2. G-CSF
3. IL-11
4. *c-kit* ligand (SCF)
5. Thrombopoietin (*c-mpl* ligand)
6. TNF (via p55 and p75 receptors)
7. IL-6
8. IL-1
9. IL-4
10. *flk2/flt3* ligand

See text for abbreviations.

Broudy et al. [9] have found receptors for stem cell factor (SCF) (*c-kit* ligand) on blasts from 20 of 20 AML patients. Both high- and low-affinity binding sites were identified, but the number of receptors per cell did not correlate with growth response to SCF. SCF was found to act synergistically with IL-3 or GM-CSF to stimulate colony growth from the marrow cells of a portion of patients with AML. Both the c-kit and GM-CSF receptor have been found to activate the Na+/H+ antiporter and to suppress apoptosis in leukemia cells [10]. Kubota et al. [11] reported that 24 out of 35 (69%) myeloid leukemia patients examined had significant c-kit-positive blast cells. The correlation between the percentage of cells positive for the c-kit molecule and the percentage of cells positive for CD34 was significant. Fifteen cases of myeloid leukemia were positive for lymphoid markers, and those with lymphoid markers had the highest expression of c-kit. SCF enhanced the colony growth in 5 of 6 AML cases tested that expressed the c-kit molecule.

mRNA transcripts of thrombopoietin (TPO) were detected in 18 of 50 AML cases and in some myeloid leukemia cell lines by RT-PCR in one study [12]. TPO was coexpressed with *c-mpl*, the ligand for thrombopoietin, in 10 of 50 AML cases. As expected, TPO and *c-mpl* were observed with high frequency in AML cases of megakaryoblastic (FAB M7) type. TPO was not found in the conditioned medium in TPO mRNA-positive leukemia cells cultivated in vitro. Matsumura et al. [13] found that recombinant human thrombopoietin was able to support proliferation of AML cells for 2–4 weeks in 4 of 15 cases that were M_0, M_2, M_4, and M_7 by FAB subtype. In two cases, some stimulation of megakaryocytic differentiation was noted. What implications this might have for clinical use of *c-mpl* ligand in AML remains uncertain with reference to the ability of this growth factor to increase proliferation of AML blasts. Fresh myeloid leukemia blast cells and myeloid leukemic cell lines also express the FLT3 receptor [14]. FLT3 ligand is a hematopoietic growth factor that plays a key role in growth of primitive hematopoietic cells.

AML blasts also express both tumor necrosis factor (TNF) receptors, namely, TNF-Rp55 and the TNF-Rp75. TNFα stimulation of AML blasts occurs through both p55 and p75 TNF receptors as assessed by receptor-specific antibody blocking. TNFα-signaled growth inhibition is mediated via

only the TNF-Rp55. TNF has been found to have multiple effects on AML blasts. It synergizes with IL-3 and GM-CSF, inhibits the effects of G-CSF and SCF in vitro, induces autocrine growth, and suppresses proliferation of clonogenic leukemic cells [15]. Khoury et al. [16] have reported that when $CD34^+$/c-kit^+ AML cells are exposed to TNFα, their proliferative response to stem cell factor (*c-kit* ligand) is decreased. The downregulation of c-kit receptors was mediated via the p55 TNFα receptor.

The expression of IL-6, IL-6 receptor, and gp130 has been examined by Inoue et al. [17] in cases of AML and acute undifferentiated leukemia. Twenty-one percent of AML patients expressed IL-6. IL-6R was expressed in all cases of leukemia tested, and gp130 was likewise ubiquitously expressed. Point mutations of *n-ras* have been associated with expression of IL-6 in AML cells [18].

Based upon the findings that AML blasts have receptors for multiple growth factors and can in certain instances proliferate in response to exogenous administration of growth factors in vitro, the simultaneous exposure of AML cells in vivo to multiple growth factors would be expected to result in either additive, inhibitory, or synergistic effects. That these effects of growth factors on AML blasts are complex and interactive is supported by work such as that reported by Wagteveld et al. [19]. They found that IL-4 enhanced proliferation in a subset of AML cells and that the combination of IL-1 and IL-4 was additive in about 50% of cases studied. It was found that IL-1 induced the endogenous secretion of GM-CSF, IL-6, and TNF in most cases, but no secretion of growth factors was induced by IL-4 alone. In some cases, however, IL-4 suppressed the IL-1-induced secretion of GM-CSF, TNF, and IL-6. In addition to the complex interactions of growth factors in vivo, there is significant heterogeneity in AML blasts as regards the expression of growth factor receptors or their ability to respond to growth factors in vitro and presumably in vivo [20].

The ability of some AML blasts to proliferate in response to growth factors led to concerns that use of pharmacological doses of growth factors might stimulate leukemic growth in various clinical situations. Despite the presence of G-CSF and GM-CSF receptors on AML blasts, growth factor administration during induction or consolidation treatment courses for AML does not seem to stimulate the growth of leukemia [21–24]. There is no evidence of stimulation of the growth of myeloid leukemia cells by G-CSF if it is used for a short period of time and if the number of leukemic cells is reduced by chemotherapy. Also, there is no evidence that GM-CSF or G-CSF administration increases relapse rates of leukemia after chemotherapy once remission is attained [22,25–27].

Growth factors currently available for clinical use can thus generally be administered safely to patients with AML. Major side effects that may be encountered are fever, rashes, capillary leak syndromes, and joint aches. These toxicities are also common when growth factors are not used [28]. When growth factors are used in leukemia, no increase in early relapse has been

demonstrated [29,30]. This finding is discussed in further detail below in the context of clinical usage of G-CSF and GM-CSF to reduce neutropenia.

Reducing period of neutropenia

Early death during induction therapy for acute myelogenous leukemia (AML) still remains an important clinical problem. The mortality rate increases with age and, for patients over 55 to 60 years, the treatment-related early death rate is on the order of 20% to 40% [31–35].

Due to the potential for cytokine stimulation of acute leukemia as discussed above [29,36–38], there was initially great hesitation in conducting clinical trials with colony-stimulating factors in acute leukemia. Much of the early work highlighted this concern for the clinical safety of cytokines in AML by elegant experiments showing blast cell proliferation as well as leukemia progenitor cell proliferation and differentiation in culture systems. It is not surprising, therefore, that when clinical trials finally did get under way, these were conducted in older patients, where the risk of death from marrow aplasia was high enough to outweigh the potential risk of stimulating leukemia cells. The first such trial was reported in 1988 [39], and since then several clinical trials using colony-stimulating factors in AML have been conducted. The first report to convincingly show that cytokines could shorten the recovery period following induction therapy was published in manuscript form by Büchner in 1991 [26]. GM-CSF was used, and it was shown that the neutrophil recovery period was shortened by approximately one week when GM-CSF was given to relapsed patients or to patients with de novo AML. Since the original study of Büchner, quite a few clinical studies have been published — some single-arm studies and, more recently, several controlled studies. Table 3 summarizes these studies, indicating the number of patients who actually received the growth factors, not the total number of patients on the study. Most studies have employed either G-CSF, yeast-derived GM-CSF, or *E. coli*-derived G-CSF. Most studies describe a major effect on neutrophil recovery, with the exception of some studies using the *E. coli* form of GM-CSF. A brief glance at Table 3 also indicates that the stimulation of leukemia — a major early concern — appears not to be a relevant clinical problem. Only one recent trial, using the *E. coli* form of GM-CSF [41], reported this to be a significant clinical problem, albeit in a small cohort of patients.

It is perhaps paradoxical that, in spite of a multitude of clinical studies evolving around the use of hematopoietic growth factors during induction therapy for acute leukemia, much controversy abounds. A closer look, however, reveals a great variability in study conditions (Table 4) and very few identical study designs. In fact, most studies may have fundamental differences in their design that impact on the results and have highlighted the confusion that surrounds this important area.

In comparing clinical trials, it is particularly important to compare both

Table 3. Select studies of cytokines in AML given during induction therapy

Study [ref]	*N*	Cytokine	Neutrophil recovery	Clinical effects	
				Reduced morbidity and/or mortality	Leukemia stimulation
Büchner, 1991 [26]	36	GM-CSF (Yeast)	+	+	−
Rowe, 1995 [22]	60	GM-CSF (Yeast)	+	+	−
Estey, 1990 [40]	12	GM-CSF (*E. coli*)	−	−	−
Zittoun, 1994 [41]	27	GM-CSF (*E. coli*)	−	−	+
Witz, 1994 [42]	103	GM-CSF (*E. coli*)	+	−	−
Stone, 1995 [21]	180	GM-CSF (*E. coli*)	+	−	−
Löwenberg, 1995 [43]	151	GM-CSF (*E. coli*)	+	−	−
Ohno, 1992 [2]	67	G-CSF	+	+	−
Baer, 1993 [44]	20	G-CSF	+	−	−
Estey, 1994 [45]	69	G-CSF	+	−	−
Dombret, 1995 [46]	88	G-CSF	+	−	−
Ohno, 1994 [47]	28	G-CSF	+	+	−
Godwin, 1995 [48]	93	G-CSF	+	−	−
Heil, 1995 [49]	240	G-CSF	+	+	−

Abbreviations: *N*, number of patients who actually received cytokine.

Table 4. Variability in the design of clinical studies

- Patient age
- Induction regimen used
- Disease state
- Timing of growth factor administration
- Documentation of marrow hypoplasia
- Differences in study product
- Statistical endpoints

patient ages and the induction regimen used. It is theoretically possible that a more intensive induction regimen, with a more profound suppression of the bone marrow, may demonstrate a greater potential for a beneficial result from cytokine use. A study by the Eastern Cooperative Oncology Group [22] used a more intensive regimen than other similar studies — 60 mg/m^2 of daunorubicin as well as seven days of 100 mg/m^2 cytosine arabinoside — and demonstrated an unequivocal benefit for the cytokine tested. Also important when considering differences in study results is the disease state, i.e., whether de novo AML or more advanced disease was present. Timing of growth factor administration may indeed be another critical issue. Several studies of cytokines in AML [2,22,26] administered the cytokine only when marrow aplasia had been demonstrated. Most of the other studies administered the cytokine at varying points, ranging from start of induction therapy to immediately following the completion of such therapy. In these latter studies, it may indeed be difficult to ascertain the effect of cytokines that one is testing, i.e.,

Table 5. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF)

Properties	Yeast (sargramostim)	*E. coli* (molgramostim)
Physicochemical	Glycosylated 127 amino acids	Nonglycosylated 121 amino acids
Pharmacokinetic	Slower rate of distribution	
Leukotriene biosynthesis	++(?)	++++
Clinical toxicity		Increased fluid retention Increased respiratory symptoms Increased fever, myalgias
? Differences in clinical activity		

whether cytokines produce a stimulatory effect on leukemic blasts — a potential positive or negative effect due to 'priming' — or stimulation of normal hematopoiesis. This question could make interpretation of the data difficult because of these separate yet possibly interactive effects.

Other considerations revolve around the use of different study products, perhaps also hindering direct comparisons, since there may be subtle differences even among different cellular preparations of the same cytokine. The statistical endpoints used in the design with many of these studies need to be very carefully considered. Some of the studies were designed and sized to show differences in hematopoietic recovery, for example, but not to show differences in complete response rates or survival. Because of the magnitude of the potential difference in days to hematopoietic recovery, the number of patients required to demonstrate such a difference is far less than the number needed to show differences in response rate or survival, which, in a typical AML study, is 300 to 400 patients. It cannot be overemphasized that in such studies, a lack of a statistically significant response rate does not demonstrate a lack of response but rather the inability to make any substantive conclusions due to the intrinsic study design.

Virtually all reports in the literature make generic references to G-CSF and GM-CSF. However, this may not be appropriate, particularly in the case of GM-CSF. Because of differences in physicochemical properties and pharmacokinetics, the Food and Drug Administration (FDA) decided to assign different generic names for GM-CSF, based on the expression system used. Sargramostim is a glycosylated GM-CSF product in yeast and molgramostim is the non-glycosylated GM-CSF product in *E. coli*. The glycosylated product has a slower rate of distribution than the nonglycosylated GM-CSF and has a high rate of leukotriene metabolism. The increased leukotriene production is thought to be related to the increased clinical toxicity seen with a nonglycosylated GM-CSF — most importantly, the increased fluid retention, respiratory toxicity, fevers, and myalgias. The differences in toxicity and physicochemistry as well as pharmacokinetics have been clearly established (Table 5) [50]. It is not known, however, whether there are any clear differ-

ences in the clinical spectrum of activity between the two products, but such differences could explain some of the conflicting results from clinical trials. One report [51] described the differences in GM-CSF serum concentrations in healthy human volunteers treated with the non-glycosylated *E. coli* GM-CSF and the glycosylated chinese hamster ovary GM-CSF. A peak effect with a more rapid increase and decrease in GM-CSF serum concentrations was evident in the nonglycosylated GM-CSF product. What was more important was that the slower rate of distribution in the glycosylated GM-CSF product corresponded to a more sustained increase in the actual leukocyte counts in these volunteer donors. This outcome clearly provides a potentially important theoretical difference that could lead to marked clinical effects, although the significance of this finding in current clinical trials has never been proven. It is also possible [51] that the functional activation of leukocytes, including the increased generation of leukotrienes, may also explain some of the adverse effects caused by GM-CSF — which, in turn, would explain the greater clinical toxicity seen with the nonglycosylated forms of GM-CSF.

It is not the intent of this chapter to describe all published trials in great detail. A study by the Eastern Cooperative Oncology Group [22] will be described as a prototype with which other studies can be compared. The reason for this choice is that this study was the first reported prospective, placebo-controlled study [52] and has shown the most clear-cut benefit for cytokines following induction therapy in AML. In this study, adult patients over the age of 55 were treated with daunorubicin 60 mg/m^2 intravenously for three days and cytosine arabinoside 100 mg/m^2 intravenously for seven days.

A bone marrow was performed three days after completion of one or two cycles of chemotherapy (day 10), and if this was aplastic, yeast-derived GM-CSF at a dose of 250 μg/m^2 (or an equal volume of placebo) was administered starting on day 11 until neutrophil recovery to 1500/μL. Those patients who went into complete remission received consolidation therapy with cytosine arabinoside 1.5 g/m^2 intravenously every 12 hours for a total of 12 doses. Patients received the same randomized study drug in consolidation as they received during induction in order to avoid any potential crossover effects.

One hundred and twenty-four patients were entered in the study, and the overall complete remission rate was 60% among patients receiving GM-CSF and 44% among those on placebo ($p = 0.07$). For those patients aged 56 to 65 years, the difference was 68% versus 48% and for those over 65, the difference was 45% versus 31% for GM-CSF and placebo, respectively. Once again, it is important to remember that this study was not sized to show differences in complete remission rate. The mortality following study drug administration (after day 11) was 6% among patients receiving GM-CSF and 15% among patients receiving placebo ($p = 0.18$), but if this finding is extended to patients receiving study drug while on the study or within 30 days of study drug completion, a very significant difference ($p = 0.015$) was seen for GM-CSF (7.7%) versus placebo (28%). Importantly, there were very significant differences in the death rate from pneumonia and from fungal infections. The

neutrophil recovery was significantly shortened among patients receiving GM-CSF, although there was no difference in the platelet or red cell recovery (Table 6).

The overall survival of all eligible and evaluable patients entered in this study is shown in Figure 1 and includes all patients who were assigned to GM-CSF or placebo, whether or not they actually receive the medication; in other words, this is an intent-to-treat analysis. The median survival of patients in the GM-CSF arm was significantly longer at 323 days versus a median survival of

Table 6. Median times from start of GM-CSF/placebo (day +11) to recovery

	GM-CSF	Placebo	*P* Value
ANC >500/μL	13d (11,16)	17d (12,40)	0.0015
ANC >1000/μL	14d (12,18)	21d (13,43)	0.0002
Platelet >20,000/μL	11d (7,18)	12d (9,80)	0.11
RBC	13d (9,36)	14d (9,78)	0.39

Median times of recovery in days (d), as well as the 25th and 75 percentiles (in parentheses).

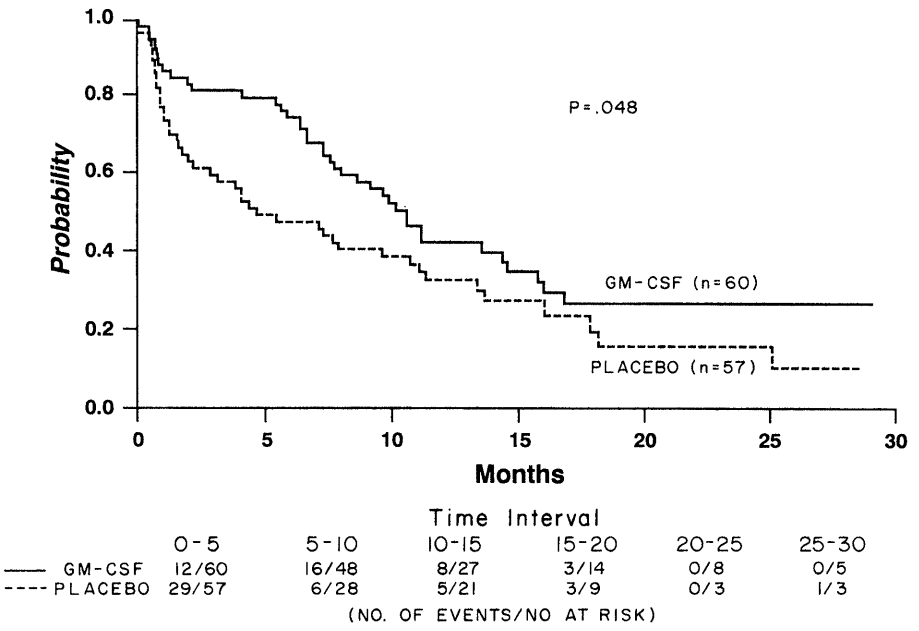

Figure 1. Overall survival of all eligible and evaluable patients entered on this study. The median survival for 60 patients randomized to receive GM-CSF was 10.6 months and was 4.8 months for the 57 patients randomized to received placebo ($p = 0.048$). The number of patients at risk at various time points are indicated. (Reprinted from Blood 86:457–462, 1995, with permission.)

145 days ($p = 0.049$) for placebo. From the slope of the curves, it seems that the difference appears to be almost entirely due to death within the first two months.

A major concern in these studies has been the fact that even if cytokines reduce morbidity and mortality, their use may lead to an increased rate of relapse. Figure 2 shows the disease-free survival of all patients in this study who entered complete remission. Essentially, the relapse rate in the GM-CSF and placebo patients are virtually identical.

Although the morbidity and mortality were significantly improved with GM-CSF in this study, the days of hospitalization were not different (36 days for GM-CSF vs. 38 days on placebo). This fact, however, is another misleading piece of information that has been frequently misinterpreted and suggested as a 'lack of benefit' for GM-CSF. Such published data represent all patients in a study and, of course, include all patients who died early from toxicity. Since there were significantly more early deaths in the placebo arm, and since these patients had a very short hospitalization, it is not surprising that the total days of hospitalization comparing all patients in this study are not different. If

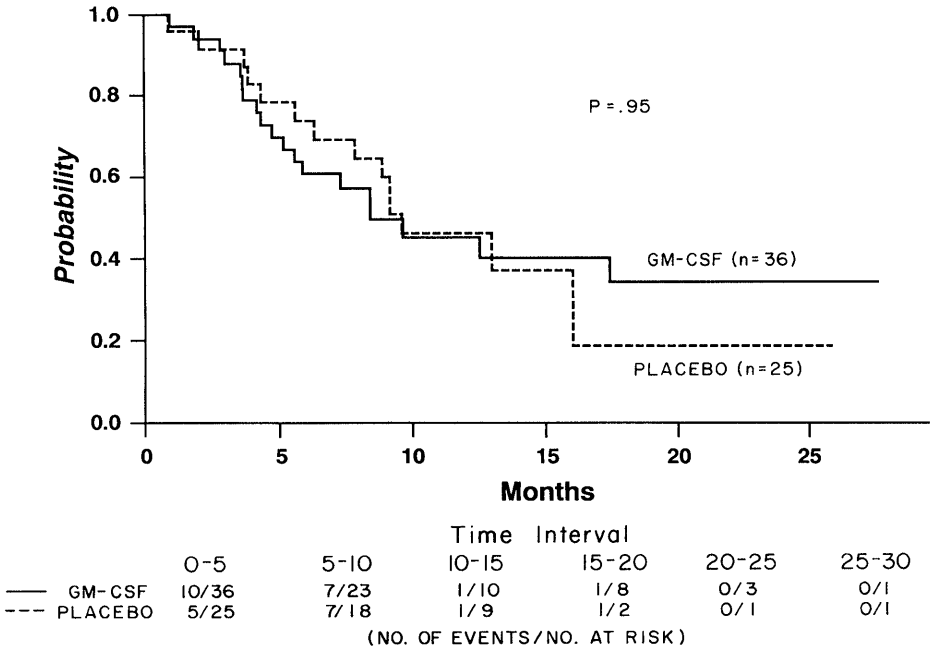

Figure 2. Disease-free survival, from time of achievement of complete remission, for all patients who achieved complete remission. There is no evidence for an acceleration of relapse in patients who received GM-CSF. The number of patients at risk at various time points are indicated. (Reprinted from Blood 86:457–462, 1995, with permission.)

indeed one looks only at patients who survived induction, the differences in hospitalization are significant.

Clearly, other studies do not show similar results. The large study by the Cancer and Leukemia Group B [21] demonstrated a small, but significant, difference in neutrophil recovery (15 vs. 17 days) in favor of GM-CSF. However, the authors describe this to be of no clinical significance, and there were no differences in the infection rate, morbidity, or mortality in the study. It is important to note that the *E. coli* form of GM-CSF was used; patients received GM-CSF without any attempt to ascertain whether marrow aplasia had been reached, and there was a significant dropout rate — approximately 30% — in patients taking study drug in both arms. The fact that this dropout occurred in both arms suggests a great degree of perceived toxicity using this particular preparation and, perhaps, a major apprehension on the part of investigators using this study drug. Other studies show puzzling and conflicting results. One large study from Europe [46] confirmed the enhanced neutrophil recovery with G-CSF and showed a statistically significant improved complete remission rate but no difference in the overall survival.

While no doubt other well-designed studies have not shown an improvement other than neutrophil recovery time [21,43,48], too little is made of the benefit from this enhanced neutrophil recovery time. In this context, it is probably also important to note that the largest trial conducted to date [49] showed, in addition to an enhanced neutrophil recovery time, also a significant improvement in morbidity with G-CSF. Table 7 summarizes the data from most of the controlled trials of growth factors following induction therapy for AML. Although the complete data are not always available, Table 7 includes as much data as could be obtained from the published information. Three separate groups are identified: the two trials using yeast-derived GM-CSF; the six published trials of G-CSF; and four published trials using *E. coli*-derived GM-CSF. Certain points are clear at a glance. Virtually all studies confirm the safety of cytokines in induction. Only one study [41] showed *E. coli* GM-CSF to have a deleterious effect, but this finding was not confirmed in any of the other large studies, and in general, the overwhelming data in several thousand patients has allayed any fears regarding the safety of cytokines during induction. Table 7 also emphasizes the great variability among the studies, e.g., in the start day of administration, whether marrow aplasia had been achieved, and in the type of cytokine used. It is also apparent from this table that virtually all studies using yeast-derived GM-CSF and G-CSF show a significantly enhanced neutrophil recovery period. The data with *E. coli*-derived GM-CSF are less impressive, with two studies [42,43] showing a clear difference, another study showing a minor, but significant difference [21], and a further study showing no difference [21]. Table 7 further indicates that most studies using yeast-derived GM-CSF or G-CSF show at least a trend towards an improved complete remission rate, and one study shows a very significant difference [46]. Two studies with G-CSF show no such difference [48,49]. No such apparent effect is shown in any of the studies using the *E. coli* form of

Table 7. Controlled trials of growth factors after induction therapy for AML

Author [Ref]	*N*	Cytokine	Start day	Marrow aplasia	CR (%)	Median days to ANC >1000/μL	Leukemia stimulation	Reduction in morbidity	Median survival
Büchner, 1991 [26]	86	GM-CSF (yeast) vs. control	12	Yes	50 vs. 32	10 vs. 16[a] 15 vs. 24[a]	NO	+	
Rowe, 1995 [22]	117	GM-CSF (yeast) vs. placebo	11	Yes	60 vs. 44	12 vs. 18[a]	No	+	323 days vs. 145 days[a]
Ohno, 1990 [2]	67	G-CSF vs. control	10–12	Yes	57 vs. 39	22 vs. 34[a]	No	+	
Ohno, 1994 [47]	58	G-CSF vs. placebo	−2	No	50 vs. 37	26 vs. 32[a]	No		
Estey, 1994 [45]	197	G-CSF vs. control	−1	No	63 vs. 53	21 vs. 34[a]	No		Not different
Dombret, 1995 [46]	173	G-CSF vs. placebo	8	No	63 vs. 32[a]	21 vs. 27[a]	No		9.2 months vs. 7 months
Heil, 1995 [49]	521	G-CSF vs. placebo	8	No	39 vs. 68	5 days[b]	No	+	
Godwin, 1995 [48]	234	G-CSF vs. placebo	11	Yes	42 vs. 49	3–4 days[b]	No		5 months vs. 9 months
Stone, 1995 [21]	379	GM-CSF (*E. coli*) vs. placebo	8	No	52 vs. 54	15 vs. 17[a]	No		8 months vs. 10 months
Zittoun, 1994 [41]	53	GM-CSF (*E. coli*) vs. control	8	No	48 vs. 77[a]	Not different	Yes		Not different
Witz, 1994 [42]	209	GM-CSF (*E. coli*) vs. placebo	1	No	63 vs. 61	22 vs. 26[a]	No		
Löwenberg, 1995 [43]	316	GM-CSF (*E. coli*) vs. control	1–8	No	56 vs. 55	26 vs. 31[a]	No		Not different

[a] Statistically significant; $p \leq 0.05$.

[b] Recovery quicker with G-CSF by 3–5 days. Dates not stated.

GM-CSF. At least four of the studies [2,22,26,49] using yeast-derived GM-CSF or G-CSF have shown a significant improvement in morbidity, and one study [22] shows a significant reduction in mortality using yeast-derived GM-CSF. None of the studies using *E. coli*-derived GM-CSF describes such an effect.

Considering that the major reason for the delay in initiating clinical trials in AML has been a concern for safety, it can be unequivocally stated that this concern has been put to rest. With the potential benefit demonstrated in so many studies and the almost universal finding of an improved neutrophil recovery time, it is not completely clear why there remains such a great hesitation to use cytokines in acute leukemia. It is difficult not to acknowledge a benefit for a patient undergoing induction therapy for AML who has an improved neutrophil recovery time of approximately one week. The sense of well-being among patients (and physicians) at the earliest sign of neutrophil recovery simply cannot be ignored, and, based on all of the above, the data would suggest that cytokines (certainly yeast-derived GM-CSF or G-CSF) should be administered to all patients undergoing induction therapy for AML who are at high risk for therapy-related morbidity and mortality.

Enhancement of antimicrobial function

Considerable in vitro work has, over the past decade, established a potential role of cytokines in the direct enhancement of antimicrobial function. This role, of course, has important implications in the treatment of patients with AML who experience long periods of treatment-induced neutropenia and its associated infectious complications.

Host defense mechanisms involving neutrophils and monocytes are thought to be important in combating fungal infections. In vitro, GM-CSF increases the phagocytic and fungicidal activity of neutrophils against *Candida albicans* and *Torulopsis glabrata* [53–55]. Similarly, in vitro exposure of peripheral blood mononuclear cells to GM-CSF increases phagocytosis of *Crytococcus neoformans* [56], and exposure of monocytes to GM-CSF in vitro results in increased killing of *Candida albicans* and *Aspergillus fumigatus* [57–60]. The mechanism of increased activity against candida and aspergillus appears to be unrelated to increased phagocytosis; rather, it appears to be due to increased oxidative metabolism and production of toxic superoxide anions by monocytes [58,60–62].

Disseminated fungal infections in acute leukemia are a major cause of morbidity and mortality at all ages. They are difficult to diagnose, particularly early in the course of a disease when therapy may be most beneficial. They occur with increasing frequency due, at least in part, to the use of broad spectrum antibiotics and the increasing use of indwelling catheters. Despite the development of new antifungal agents, amphotericin B remains the mainstay of therapy and, in general, recovery is associated with resolution of immunosuppression. While the high rate of mortality from disseminated

fungal infections has been clearly established, precise data are difficult to obtain, and careful consideration must be given to fundamental differences in published reports. Among other things, mortality varies with the time to neutrophilic or monocytic recovery as well as whether or not there has been documented organ involvement. It appears that in documented fungal infections during neutropenia, the reported mortality ranges from 40% to 100% in acute leukemia and is still higher in the allogeneic bone marrow transplant recipient [63]. Thus the scope of the problem cannot be overemphasized. Prevention of fungal infections during the course of therapy for acute leukemia remains critically important.

Table 8 summarizes the cytokines for which significant in vitro data suggest important antifungal action on the effector cells, specifically neutrophils and monocytes. Besides GM-CSF and M-CSF, there have been some interesting data published with the use of interferon-gamma in this regard (the Chronic Granulomatous Disease (CGD) Study Group, 1991 [64]). Other cytokines with potential antifungal activity, although probably less important, are IL-1, IL-2, IL-3, IL-4, IL-6, and G-CSF.

GM-CSF is likely to play a central role in potential cytokine activity against fungal infections. There are data that suggest that the activity of M-CSF is predominantly mediated through the release of cytokines such as G-CSF, IL-6 and, most importantly, through activity on monocytes that directly releases GM-CSF which, in turn, acts on the effector monocytes and neutrophils as well as secreting TNFα [65,66].

At the same time, it is important to recognize that macrophages may in fact be a double-edged sword in the immune system. While they clearly are a major effector arm of the cellular immune system and are recognized to be critical in antifungal immunity, they are also the principal target cell for replication of many viral, bacterial, fungal, and other pathogens and, in fact, mediate much of the damage in inflammatory diseases. There are data that suggest that macrophages play a major role in the pathology of fungal disease [67]. It is therefore difficult to predict from in vitro data whether administration of GM-CSF or M-CSF, acting directly on macrophages, will in fact benefit or exacerbate a given disease. It is likely that clinical results may depend on a delicate balance of the above activities related to specific pathogens, timing of administration, and dosage.

Table 8. Cytokines with potential direct antimicrobial activity

Cytokines	Effector cells
GM-CSF	Neutrophils
M-CSF	
IFN-γ	Monocytes
Probably less important: IL-1; IL-2; IL-3	
IL-4; IL-5; IL-6	
G-CSF	

Unfortunately, in spite of fairly abundant data, there has been no definitive clinical experience to date. Although there are some encouraging phase I or phase II clinical studies, specifically directed clinical studies are lacking, and there is a clear need for such prospective evaluations.

An early study from the M.D. Anderson Cancer Center [68] reported on eight patients with AML, ALL, and bone marrow transplant who had candida, aspergillus, and trichosporon. GM-CSF was administered as well as amphotericin B, and while these authors reported a direct increase in the neutrophil counts, the conclusions were necessarily limited.

One of the earliest and most promising phase I studies was conducted in 1991 [69], and these authors reported on 24 patients, mostly with candida, but also some with aspergillus. Patients received M-CSF together with amphotericin B. The neutrophil count, but not the monocyte count, appeared to increase, and there was an apparent improvement in 6 of 12 evaluable patients.

The CGD study group (The Chronic Granulomatous Disease Study Group, 1991 [70]) conducted a phase III study and reported on 130 patients who were treated with interferon gamma or placebo. A significantly lower infection rate was observed among patients receiving interferon gamma versus those receiving placebo. There was a marked reduction in the rate of skin and soft tissue infection, but it was also noted that aspergillus pneumonia occurred in four patients receiving placebo and one receiving interferon gamma.

In the previously reported ECOG prospective study of GM-CSF during induction therapy for AML [22], improved morbidity and mortality were noted with GM-CSF use. Specifically, the mortality from fungal infections among all patients receiving GM-CSF was 1.9% compared with 19% among those receiving placebo ($p < 0.001$). More importantly however, as can be seen in Table 9, a detailed breakdown of the type of organisms that were involved shows that the primary fungal infections were due to aspergillus and candida, as expected. Table 9 emphasizes the overall mortality among those patients who actually had a documented fungal infection. For patients receiving GM-CSF, the overall mortality from fungal infections was 13%, and among those receiving placebo, the overall mortality was 75% [71]. Basically,

Table 9. Mortality from fungal infections (phase III ECOG study)

	GM-CSF ($n = 52$)	Placebo ($n = 47$)	
Aspergillus ($n = 11$)	25%	71%	
Candida ($n = 7$)	0%	75%	
Others ($n = 2$)	0%	100%	
Overall ($n = 20$)	13%	75%	$p = 0.02$

most patients who received GM-CSF survived the fungal infection, and most patients with placebo did not. The experience among the placebo group is mostly in accordance with the common clinical experience among older adults, in which the majority of patients with a documented fungal infection during the neutropenic phase of induction therapy will not survive this. In evaluating the data from this ECOG study, it is important to emphasize that there were no differences in the treatment among the two groups, including antifungal prophylaxis. At the same time, it is important to note that this ECOG study described 20 patients with documented fungal infections, and this number must be considered relatively small to make definitive conclusions as yet. One of the interesting observations from this ECOG study has been the shortened period of neutropenia associated with the reduction in fungal infections. It is clearly not known whether direct antifungal activity of GM-CSF contributed to this outcome or whether the reduced mortality was simply a reflection of enhanced neutrophil recovery.

Prospective randomized studies are clearly needed to determine the precise prophylactic, as well as therapeutic, antifungal role of these cytokines in patients at high risk for invasive fungal infections. Because it may be difficult to differentiate a direct antimicrobial role from enhancement of neutrophilic and monocytic recovery, a patient population that is severely immunocompromised, but not neutropenic or monocytopenic, must form the basis of such prospective studies. One such group may be the postneutropenic patients with AML or ALL who have opportunistic infections such as hepatosplenic candidiasis or recipients of allogeneic bone marrow transplantation who remain severely immunocompromised, but are not neutropenic or monocytopenic. Clearly, this area has great potential for direct cytokine activity, and it is hoped that, over the next decade, much progress will be made in this area.

Recruitment of cells into the S phase of the cell cycle

Several investigators have examined the issue of whether hematopoietic growth factors can be utilized to prime leukemic blasts by increasing the proportion in the active phase of the cell cycle, thereby, theoretically, increasing their susceptibility to S-phase active agents such as cytosine arabinoside. In vitro studies have shown that growth factors such as GM-CSF, stem cell factor (*c-kit* ligand), interleukin-3 (IL-3), and others can increase the fraction of leukemic blasts in S phase [72]. Also, it has been found that combined exposure of AML blasts to cytosine arabinoside and GM-CSF may enhance the intracellular uptake of Ara-CTP, thereby increasing antileukemic activity [73]. In normal hematopoietic cells, GM-CSF results in increased uptake of dCTP rather than Ara-CTP [74]. Whether this results in differential toxicity to leukemic progenitors remains uncertain.

The role that in vivo priming might have on leukemic blasts and ultimate

chemotherapy remains uncertain. Some of the earlier studies [75] have suggested that priming with GM-CSF can increase disease-free survival rates, but other studies have not shown this [76].

It is important to emphasize at the onset that in vitro data must be cautiously interpreted when they are described without concurrent controls. The reporting of an increased number of blasts or recruitment into the S phase of the cell cycle following administration of a cytokine needs to be carefully compared with these statistics in control patients, since patients with AML at presentation who are left with no therapy will often both change their cell cycle kinetics and increase their blast cell count [77–79].

One of the earliest clinical reports on the use of cytokines to prime leukemic cells was a description in 1991 [80] of the effects of GM-CSF on leukemic cell cycle progression and cell kill with cytosine arabinoside. Following administration of GM-CSF, a threefold increase in the fraction of blasts in the S phase was noted.

Probably a milestone in the clinical development of the concept of priming was the pioneering work of Bettelheim et al. [81], in which 18 patients with AML were treated with GM-CSF in combination with cytosine arabinoside and an anthracycline. The GM-CSF, in most patients, was started 24 to 48 hours prior to chemotherapy, and in vitro studies showed recruitment into the S phase in all patients. Most importantly, the aplasia was not prolonged, and two thirds of patients went into complete remission after one cycle, with no evidence of stimulation of leukemic clonogenic blast cells. This early study surprised many and appeared to confound some of the concerns and predictions in that there appeared to be no deleterious effect of GM-CSF when administered prior to induction therapy and when it clearly was shown to stimulate the leukemia cells.

Multiple in vitro studies have reported on GM-CSF being given to AML patients either concurrently or just prior to induction therapy. Most of these studies have demonstrated an increase in the percentage of cells in the S phase [78,82–84]. Furthermore, another report [85] demonstrated an enhancement of cytosine arabinoside cytotoxicity by both GM-CSF and G-CSF as well as enhanced promotion of cytosine arabinoside incorporation into DNA as a result of an increased cell fraction in the S phase. In this study, the AML-19 human leukemia cell line was used, since this cell line requires GM-CSF or G-CSF for optimal growth.

On the other hand, studies on apoptosis [86] demonstrated that transforming growth factor beta-1 (TGFβ1) induces cell death in myeloid leukemia by apoptosis. Importantly, the apoptosis-induced in leukemia cells by TGFβ1 was inhibited by G-CSF, GM-CSF, or IL-3. Of major concern was the demonstration that similar inhibition of the apoptosis induced by common cytotoxic agents such as vincristine, adriamycin, and cytosine arabinoside could be observed using G-CSF or GM-CSF. Basically, this worrisome observation suggested that G-CSF and GM-CSF prevented one of the important mechanisms of control of leukemia. These authors cautioned that the clinical use of

cytokines to correct therapy-associated myeloid suppression needs to be carefully timed to avoid protection of the malignant cells from the cytotoxic actions of the therapeutic compounds.

Since the pioneering work of Cannistra and Bettelheim [80,81], several studies have been performed on cytokines used as a priming agent for AML. These are outlined in Table 10. The table clearly shows the enormous variability in the patient population, the cytokine used, the types of therapies administered, and, of course, the result. Nevertheless, it is important to note that, apart from one report [76], most controlled trails have confirmed the earlier pilot data of Cannistra and Bettelheim [80,81] that indicated that stimulation of leukemia is not an important clinical problem. These studies also demonstrated that, despite reasonable concerns, prolonged aplasia due to susceptibility of residual normal progenitors to cytotoxic chemotherapy does not appear to be an important feature.

It is perhaps important to discuss briefly the one study that demonstrated stimulation of leukemia and a lesser response rate among patients receiving *E. Coli*-derived GM-CSF [76]. In this study from M.D. Anderson (Houston, TX, USA) 56 patients with de novo AML were treated with GM-CSF prior to induction chemotherapy using two different protocols. In the first protocol, GM-CSF was administered at a dose of 125 $\mu g/m^2$ starting seven days before induction chemotherapy, and in the second, older individuals (over 60) received 20 $\mu g/m^2$ for four days followed by a dose of 125 $\mu g/m^2$ for four additional days — in other words, the priming therapy was started eight days prior to chemotherapy. The growth factor was continued until chemotherapy was completed. The outcome of these data were compared with historical controls for patients receiving induction therapy without GM-CSF. However, just as the patients with GM-CSF were treated in two different studies, so the comparison historical-control patients were also taken from two different studies, with patients being treated with cytosine arabinoside with or without amsacrine or mitoxantrone. Thus, data from this study need to be very cautiously interpreted, especially in light of the accumulating data suggesting that the safety of priming with cytokines appears not to be a major clinical issue.

Nevertheless, the issue remains open, and the data are conflicting and confusing. Clearly, large prospective randomized studies must be performed before any conclusions can be drawn. At the present time, the Eastern Cooperative Oncology Group (ECOG) is close to completing a prospective double-blind study where patients receive yeast-derived GM-CSF or placebo 48 hours prior to initiation of standard chemotherapy for older adults with AML. Bone marrow and peripheral blood is examined prior to initiation of the priming therapy and again 48 hours later. Cytokine receptors as well as cell cycle studies and pharmacokinetics are evaluated on each occasion, and the study drug (GM-CSF or placebo) is continued until marrow aplasia is achieved, at which point all patients receive open-label GM-CSF based on the ECOG data previously reported [22].

At the present time, unlike the use of cytokines to shorten the period of

Table 10. Results of studies of cytokine used as priming therapy for AML

Authors [Ref]	*n*	Cytokine	Day of administration	Leukemia stimulation	CR (%)	Survival
Bettelheim, 1991 [81]	57[a]	GM-CSF (yeast)	−2	No	83	Not stated
Cannistra, 1991 [80]	3	GM-CSF	−2	No	33	Not stated
Estey, 1992 [76]	232[a]	GM-CSF (*E. coli*)	−8	Yes	48	Worse with GM-CSF
Estrov, 1992 [87]	14	GM-CSF (*E. coli*)	−2	No	71	Not stated
Valent, 1992 [88]	59[a]	GM-CSF (*E. coli*)	−1	No	85	Not stated
Büchner, 1993 [75]	48[a]	GM-CSF (yeast)	−1	No	81	2-year disease-free survival 39%
Archimbaud, 1993 [82]	58[a]	GM-CSF (yeast)	−2	No	30	64 days vs. 129 days (control)
Estey, 1994 [89]	197[a]	GM-CSF (*E. coli*)	−1	Yes	63	Not different
Visanti, 1994 [90]	28	G-CSF	−1	No	58	Not stated
Ohno, 1994 [47]	27[b]	G-CSF	−2	No	50	Not different
Zittoun, 1994 [41]	51[a]	GM-CSF (*E. coli*)	−1	No	72	Not different
Yamada, 1995 [91]	18	G-CSF	−2	No	83	17 months median
Witz, 1995 [92]	209[b]	GM-CSF (*E. coli*)	1	No	63	2-year disease-free survival (44% vs. 19%); $p < 0.05$
Frenette, 1995 [83]	44[a]	GM-CSF (*E. coli*)	−1	No	37	9.5 months median
Peterson, 1996 [93]	174	GM-CSF (*E. coli*)	−5	No	33	

[a] includes historical controls.

[b] includes placebo controls.

neutropenia, it seems that the use of GM-CSF or G-CSF as priming therapy should not be recommended outside of well-designed clinical studies. Although most studies have not demonstrated beneficial clinical effect, this result does not rule out the possibility that certain subtypes of patients may benefit from such therapy. A recently completed study by the Cancer and Leukemia Group B (CALGB) [90] also demonstrated no clinical benefit, but although this study was placebo controlled, the dose of *E. coli*-derived GM-CSF was 3 μg/kg, arguably too low to elicit a priming effect.

Induction of differentiation of leukemic cells

In certain cases, cytokines might have the ability to differentiate leukemia cells or progenitors. This has been found to be the case in the leukemic cell line HL60 and in some murine models of leukemia [94] where IL-6 resulted in an increase in mature cells in marrow and a decrease in blasts and promyelocytes.

Some clinically available growth factors such as G-CSF and M-CSF may induce differentiation of leukemic progenitors, but there is wide heterogeneity in these responses [95,96]. G-CSF, GM-CSF, and IL-3 have been utilized in the treatment of myelodysplasia with some increase in neutrophil counts, suggesting a differentiation effect. It remains unclear in these cases whether the leukemic clone is being stimulated to differentiate or whether residual normal progenitors are being stimulated to differentiate.

Differentiating cytokines have not seen extensive examination in clinical settings. Using M1 myeloblastic leukemic cells as a model system, cytokines such as IL-6 induce terminal differentiation associated with growth arrest, whereas negative regulators such as TGFβ1 induce rapid growth arrest and apoptosis [97]. How differentiation programs interact with programmed cell death pathways remains to be determined. Many cytokines that induce differentiation of culture-adapted cell lines do not induce differentiation of fresh AML blasts [98].

Direct Antileukemic effects of cytokines

Most cytokines that might have direct antiproliferative effects on AML blasts have not yet seen widespread clinical trials (Table 11).

IL-6

Suzuki et al. [99] examined the combined effects of five cytokines (G-CSF, GM-CSF, IL-1β, IL-3, and IL-6) on blast colony formation by leukemic blast progenitors from 10 patients with AML. While the combination of G-CSF, GM-CSF, IL-1β, and IL-3 stimulated maximum blast colony formation in nine

Table 11. Cytokines with direct antileukemia effects

1. IL-6
2. G-CSF
3. MIP-1α
4. IFN-α
5. IL-10
6. TGF-β

patients, addition of IL-6 reduced the stimulatory effect. Interleukin 6 alone has also been reported to inhibit AML in mice and in cell culture [100].

While IL-6 may have an inhibitory effect on proliferation of AML cells in vitro, no responses were seen in a phase II study where eight patients with AML in first relapse were given IL-6. [101]. These patients had 5%–29% marrow leukemic blasts. In addition to a direct antileukemic effect, IL-6 may be associated with downregulation of the apoptosis-inhibiting gene bcl-2. Decreased expression of bcl-2 may increase sensitivity of the blasts to apoptotic cell death by chemotherapeutic agents [102].

G-CSF

In the postallogeneic bone marrow transplantation setting where relapse of acute myelogenous leukemia has occurred, G-CSF has been found to cause disease remission, both hematologic and cytogenetic, with reestablishment of hematopoieisis of donor origin in 3 out of 7 cases [103]. The mechanisms responsible for this have not been well described.

MIP-1α

Macrophage inflammatory protein-alpha (MIP-1α), has been found to significantly inhibit primitive and mature AML progenitors up to 79% with sample-to-sample variability. This outcome could be seen in the presence of GM-CSF, G-CSF, or IL-3. MIP-1 prevented entry of AML blasts into the proliferative phases of the cell cycle and had no effect on IL-1β levels [104].

IFN-α

Inteferon-alpha has been found to be a potent inhibitor of AML cell proliferation in vitro [105].

IL-10

Interleukin-10 has been found to inhibit spontaneous AML blast proliferation in a majority of patients, whereas when cultured in the presence of other

exogenous growth factor, IL-10 demonstrates variable effects [106]. The effects of IL-10 appeared to be irreversible. Also, IL-10 decreased secretion of IL-1α, IL-1β, TNFα, GM-CSF, and IL-6. Apoptosis and functional status (differentiation) of AML blasts were not affected.

TGFβ

TGFβ is discussed below in Stem Cell Protection section.

Interruption of autocrine/paracrine loops

Blast cells from 70% of patients with AML show evidence for autonomous growth, which may relate to the autocrine secretion of growth factors, especially GM-CSF [107]. Patients with autonomous blast growth have a lower remission rate and a higher relapse rate [108]. AML blasts with autonomous growth are thought to be relatively resistant to programmed cell death (apoptosis), and this may be related to the autocrine production of GM-CSF and its upregulation of *bcl-2* expression. This increased *bcl-2* expression is thought to protect cells from apoptosis induced by cytotoxic drug therapy [109]. Bradbury et al. [110] found that blasts with non-autocrine growth rapidly lost viability following 48 hours of culture due to the onset of apoptosis. In these cells, GM-CSF prevented apoptosis, and *bcl-2* was upregulated. Blasts from patients with autonomous growth and autocrine GM-CSF production did not demonstrate apoptosis, and in these cells neutralization of GM-CSF downregulated *bcl-2*.

In the majority of cases of AML that demonstrate autonomous growth, neutralizing antibodies to the relevant cytokines inhibit growth, but in some cases, there is evidence for intracellular autocrine loops [107]. This can be demonstrated by antisense oligonucleotides, which inhibit GM-CSF DNA or mRNA transcription or translation in cases where an internal GM-CSF autocrine loop is in place. In other cases of AML, paracrine stimulation of leukemic growth may also be operable, but precisely which cells are involved (e.g., accessory lymphoid or monocytic cells or stromal cells of the marrow microenvironment) has not been well investigated in vivo. Interruption of both autocrine and paracrine pathways by antibodies to growth factors or their receptors, by soluble growth factor receptors, by growth factor receptor antagonists, or by antisense oligonucelotides represents possible mechanisms to control leukemic cell growth. Few studies in patients are reported, but there is much in vitro work to confirm that such approaches may have utility (Table 12).

Interleukin-1 has been reported to play an important role in AML blast proliferation and may be part of autocrine growth mechanisms. Freshly obtained leukemic blasts have been shown to spontaneously express the IL-1 gene and to secrete interleukin-1. Some leukemic blasts will proliferate in

Table 12. Mechanisms to interrupt autocrine stimulation of AML blasts

1. Neutralizing antibodies to autocrine growth factor(s) involved
2. Neutralizing anti-growth factor receptor antibodies
3. Soluble growth factor receptors
4. Growth factor receptor antagonists
5. Antisense oligonucleotides (to inhibit growth factor DNA or mRNA expression)

response to IL-1, and many have IL-1 receptors. Anti-IL-1 antibodies can inhibit the spontaneous proliferation of leukemic blasts [111]. Estrov et al. [112] examined the effect of soluble IL-1 receptors (sIL-1R) and IL-1 receptor antagonist (IL-1RA) on the growth of AML marrow progenitors from 25 patients. IL-1RA competes with IL-1 for receptor binding, but it does not activate the receptor as does IL-1. In blast colony assays, both substances inhibited blast colony formation, and this inhibitor effect was partially reversed by IL-1β. This inhibition by sIL-1R or IL-1RA could also be demonstrated in the presence of fetal calf serum or various CSFs such as GM-CSF, G-CSF, IL-3, and *kit* ligand. Also, incubation of AML cells with sIL-1R or IL-1RA has been found to reduce the concentration of GM-CSF or G-CSF in lysates from patient samples [112].

Stosic-Grujicic et al. [113] have reported that a 16-mer phosphorothiorate oligodeoxynucleotide against IL-1β converting enzyme (ICE) inhibited spontaneous as well as induced CFU-AML colony formation, with sample-to-sample variability noted. The same group also reported that IL-1 receptor antagonist inhibited spontaneous proliferation of blast cells from 9 of 14 patients, but at lower doses, it induced potentiation of spontaneous proliferation, suggesting another regulatory pathway for IL-1RA. Whether IL-1 inhibitory molecules will have a role in future treatment of AML remains to be determined.

IL-1 may stimulate leukemic blast proliferation by inducing GM-CSF mRNA and protein expression. Bradbury et al. [114] reported that blasts with totally autonomous growth secreted the highest levels of GM-CSF and IL-1β. Antibodies to IL-1 can suppress GM-CSF expression, and antibodies to GM-CSF can decrease response to IL-1. IL-1 also stimulates G-CSF, GM-CSF, and M-CSF expression in stromal and endothelial cells, so it may indirectly stimulate leukemic blasts in vivo via this mechanism [115,116]. Bruserud [117] has reported that while endogenous IL-1 secretion varies widely between individual cases of AML, inhibition of IL-1 decreases blast secretion of IL-1α, G-CSF, GM-CSF, TNFα, and IL-6. In the presence of exogenous growth factors, the effects of IL-1 inhibition on blast proliferation are variable. The ultimate effect of IL-1 on AML blasts may therefore be dependent on the cytokine network in the AML blast microenvironment in vivo.

Interleukin 6 has also been associated with autonomous growth of AML blasts in vitro. Forty percent of AML blasts with autonomous growth have

been reported to exhibit abnormalities of retinoblastoma (Rb) protein expression. The Rb protein is a known transcriptional repressor of the IL-6 promoter, and the expression of IL-6 has been found to be significantly associated with loss of Rb protein expression [118]. Blasts from four patients who secreted high levels of IL-6 exhibited autonomous growth in vitro which could be partially suppressed by antisense oligonucleotides to IL-6.

Murohashi et al. [119] have reported that a specific neutralizing antibody against TNF-α was able in vitro to reduce the growth-stimulating effect of IL-3 on the blasts of 11 of 14 patients. The effect of anti-TNFα was greater than that of anti-IL-1α and anti-IL-1β. Constitutive TNFα transcript was observed in five of these patients, and TNFα protein was present in culture supernatant. Anti-TNFα inhibited the accumulation of TNFα transcript, suggesting that membrane-integrated TNFα may be partly responsible for the induction of the TNFα mRNA, another possible autocrine mechanism with potential effects on AML blasts.

To assess whether autonomous growth characteristics have any clinical relevance in AML, Hunter et al. [120] compared the CR rates in those patients whose leukemic cells exhibited either partial or totally autonomous growth in a blast cell colony assay. Patients whose blasts exhibited autonomous growth in vitro had a significantly lower CR rate (57%) compared with the patients whose blasts had nonautonomous growth (94%). The widespread constitutive cytokine expression by leukemic blasts may therefore play a role in driving the leukemic process [121].

Stem cell protection

Both IL-1 and TNF have been reported to protect normal progenitors from the effects of 4-hydroperoxycyclophosphamide, a cyclophosphamide cogener utilized in marrow-purging protocols [122]. Leukemic cell lines or fresh leukemic samples were not conferred this protection by TNF or IL-1 [122,123]. Estrov et al. [124] have reported that suramin, a polysulfonated naphthylurea, may inhibit the interaction between IL-1 and its receptor, thereby interrupting the autocrine and paracrine growth-stimulatory mechanism of AML cell proliferation. Suramin also inhibited normal early and mature hematopoietic progenitors; however, the suppression of normal colonies (pre-CFUs and CFU-GM) was 45% of control vs. 8% of control for AML progenitors. IL-1β inhibited the effect of suramin on AML progenitors but not on normal progenitors. This agent could therefore have an antiproliferative effect in AML patients with relative sparing of normal progenitors.

Bhalla et al. [125,126] have reported that IL-3 plus GM-CSF or PIXY-321, IL-3/GM-CSF fusion protein, can improve the selectivity of cytarabine and paclitaxel in vitro against AML blasts.

TGFβ1 has also been found to have differential effects on normal and leukemic human hematopoietic cell proliferation [127]. Taetle et al. [128] have

reported three growth patterns with TGFβ1 seen with AML cells: sensitivity to growth inhibition, resistance, and factor-dependent resistance. Some effects of TGFβ on AML cells occur through stimulation of apoptosis. In AML, TGFβ1 almost completely abolished the growth stimulated with G-CSF, whereas in normal hematopoietic progenitors, the growth stimulated by G-CSF was unaffected. TGFβ1 was also found to inhibit mRNA expression by AML blasts for c-myc and/or c-myb. It was suggested that growth suppression by TGFβ1 appeared to increase with the progression of clonal evolution in hematologic malignancies.

MIP-1α has also been reported to decrease the entry of early hematopoietic progenitors into the cell cycle, while having no similar effect on the cycling status of various leukemic cell populations. This finding may also suggest a possible use for this cell cycle chemokine as a stem cell protectant [129].

Summary

Over the past decade, with the advent of hematopoietic growth factors, major strides have been made and multiple studies have attempted to define the use of these cytokines in acute leukemia. It is perhaps disappointing that, after so many studies, so many questions remain. Nevertheless, the role of cytokines in induction therapy seems to be established, although questions remain around the issue of priming therapy. Intriguing data regarding the potential for enhancing antimicrobial function should hopefully be resolved over the next few years. What is perhaps most reassuring is that the issue of safety, which for a considerable period of time precluded the development of clinical trials in acute leukemia, has been firmly laid to rest. The use of growth factors to protect normal stem cells during treatment of leukemia and to induce leukemic cell differentiation has not yet been the subject of many clinical trials. Also, growth factors are likely targets for the interruption of autocrine leukemic blast or progenitor cell growth, but again, few clinical observations are published. With the ongoing cloning of new growth factors active both in normal hematopoiesis and in leukemogenesis, the role of growth factor use in the treatment of AML will likely be the basis for much future preclinical and clinical activity.

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9. Novel treatment approaches for cutaneous T-cell lymphoma

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A number of efficacious therapeutic approaches to the various stages of mycosis fungoides (MF) and the Sezary syndrome (SS) are available. Considerable controversy exists, however, regarding the curability of this indolent disease process with currently described modalities. The lack of convincing survival benefit to patients in randomized trials of aggressive multimodality therapy compared with palliative treatments suggests that long disease-free remissions may be observed after therapy but reflect disease biology rather than inherent curative potential of the modality applied [1].

Thus, it remains an important goal to develop new agents and approaches with unique mechanisms of action and toxicity spectra. In this way, either better cosmetic and symptomatic palliation may be achieved for the patients with this disease or, ultimately, curative treatments may be identified. In this chapter, we will outline a variety of investigational therapies that are promising based on initial clinical trial results.

Retinoids

The compounds discussed in this chapter that have given rise to the greatest clinical experience are vitamin A and its analogues. These naturally occurring compounds influence differentiation and proliferation of a number of structures during development [2], and some compounds may alter immune responses [3]. A number of approved formulations have demonstrated clinical efficacy in MF and SS.

Treatment with isotretinoin (13-*cis* retinoic acid), a nonaromatic retinoid, has been associated with clinical benefit in a number of trials. Overall objective responses have been described in 33 of 56 patients treated in three clinical trials [4–6]. A monoaromatic retinoid compound, etretinate, did not achieve similar results when tested as monotherapy for MF in several trials [7,8], but subsequently a number of responses were observed in a trial for parapsoriasis en plaque (a precursor lesion to MF) [9]. Finally, a polyaromatic retinoid (Ro 13-6298) has demonstrated activity in a small trial. Objective responses were

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observed in 3 of 6 patients (one complete response and two partial responses). All-*trans* retinoic has also been utilized in a trial for relapsed or refractory MF [10]. In a preliminary report, there were four partial responses in 13 patients.

In all these trials, investigators frequently noted significant clinical response, with resolution of lesions and relief of pruritus but persistence of atypical lymphocytes in skin biopsies. This pattern of response is atypical and suggests that the mechanism of action of retinoids may not be antiproliferative. A variety of nuclear receptors exist for the different retinoids, and the disparate binding patterns of the different retinoids may explain the wide variability of response rates recognized with one compound versus another. Also possible is that only a subset of MF/SS patients express retinoid receptors with the ability to bind retinoids and affect gene transcription. These theories await experimental confirmation.

Although the exact mechanisms of action remain unknown, the relatively favorable toxicity profile has led to a number of combination modality trials utilizing retinoids. A number of small phase II trials have utilized retinoids together with interferons; all report response rates of approximately 40%–50% [11–13]. These response rates are not clearly different than the expected response rates with interferon alone; randomized trials will be needed to determine if combination therapy is more effective than single-agent therapy. A single nonrandomized trial of retinoids (etretinate) plus PUVA in MF patients suggested a similar rate of response compared with PUVA alone (73% versus 72%) [14]. However, remissions were induced more rapidly with combination therapy, and total UVA dosage to remission was lower in the combined arm. Thus, there may be some advantages to utilizing retinoids with other active agents to enhance the effect or perhaps maintain the effects of treatment with less toxicity. The precise role of retinoids, however — alone or in combination — remains to be defined.

Interleukin-2

Interleukin-2 (IL-2) is a cytokine produced by activated T lymphocytes that results in further activation, attraction, and proliferation of T lymphocytes. Given the actions of IL-2 in vitro and in vivo, it would seem that this agent would be contraindicated in the therapy of MF or SS. However, there are now several reports of benefit with this agent. One patient experienced a partial response with intralesional interleukin-2 [15]. A second patient with diffuse skin tumors and refractory to multiple interventions was treated with continuous intravenous infusions of IL-2 every 14 days and developed a durable pathologic complete remission [16]. At Northwestern University we have also treated several patients with IL-2 with clear evidence of tumor response, but side effects were fairly dramatic, predominantly related to capillary leak with peripheral edema formation.

Purine analogue therapies

A relatively new class of agents, the purine analogues has been now developed. These agents initially were shown to be most active in hairy cell leukemia. All the agents in the class, however, have also been shown to have significant activity in clinical trials against MF/SS.

The first agent to be developed was deoxycoformycin (DCF), a transition state inhibitor of adenosine deaminase. In several small studies using DCF as a single agent at doses ranging from 4 to 10 mg/m^2 daily for three doses in patients with advanced MF or SS, the overall response rate was 41% (range 33%–67%), with complete response rates of 7% to 11% [17,18]. Several durable complete responses lasting 7 to 9 months were reported, and toxicities included renal failure, myelosuppression, and neurotoxicity.

An NCI phase II trial alternating cycles of DCF at a dose of 4 mg/m^2 for three days with intermittent high-dose interferon reported a similar response rate of 41% with a median time to progression of 13.1 months [19]. Of note, two Sezary patients had a complete response, and one of these has been in unmaintained remission for more than five years.

Fludarabine, the fluorinated derivative of vidarabine (Ara A), was the next purine nucleoside analogue to demonstrate clinical activity in MF/SS. Unlike DCF, fludarabine resists deamination by adenosine deaminase and is phosphorylated to F-ara-ATP, which inhibits DNA synthesis and repair. Von Hoff [20] treated 33 patients with MF categorized as good risk (patients with no prior systemic therapy) or poor risk (patients who had prior systemic therapy) with fludarabine at doses of 25 mg/m^2/day for five days (good risk) or 18 mg/m^2/day for five days (poor risk) and reported one complete and five partial responses. Neutropenia and thrombocytopenia were the major toxicities.

Only one study has combined fludarabine with another agent in patients with MF/SS. In this NCI phase II trial [21], fludarabine (25 mg/m^2/day $\times$ five days) was administered with concurrent low-dose interferon alpha (5 million units/m^2 three times per week). A response rate of 51% was reported, with four (11%) complete responses and 14 partial responses in a group of heavily pretreated patients. Three of the complete responders had SS and one had tumor-stage MF. The median time to progression was 5.8 months, shorter than that reported for the combination of DCF and IFN, but three of the four complete responders had unmaintained remissions for 18+, 20+, and 35+ months.

The most significant toxicities seen with fludarabine and DCF include neurotoxicity and immunosuppression. Table 1 compares the two NCI studies, namely, those of DCF-IFN and fludarabine-IFN. There was a 15%–17% incidence of sepsis and a 5%–14% incidence of opportunistic infections, including disseminated toxoplasmosis, cytomegaloviral infection, *Pneumocystis carinii* pneumonia, atypical mycobacterial infection, and fungemia in these patients, likely due to both immunosuppression related to purine analogue therapy and

Table 1. Comparison of various purine nucleoside therapies combined with interferon

	DCF-IFN	Fludarabine-IFN
Response		
Complete	2 (5%)	4 (11%)
Partial	15 (36%)	14 (40%)
Progression-free survival	13.1 mo.	5.9 mo.
Median survival	15.8 mo.	19.6 mo.
Response in patients without prior chemo or TSEB	8/12 (66%)	7/13 (54%)
Grade 3–4 bone marrow toxicity	15/41 (36%)	21/35 (60%)
Sepsis	6/41 (15%)	6/35 (17%)
Opportunistic infections	2/41 (5%)	5/35 (14%)
Grade 3–4 neurotoxicity	7/41 (17%)	5/35 (14%)

impaired underlying immune function. In addition, 14%–17% of patients developed severe neurotoxicity in the form of confusion, motor weakness, paraesthesias, and central nervous system demyelination. A recent review documents the spectrum of neurotoxicities associated with purine analogue therapy [22]. These results suggest that patients treated with these agents should be carefully evaluated for infectious complications, especially opportunistic infections, and that prophylactic therapy should be considered during and following therapy if significant immunosuppression is documented.

The most recently developed purine analogue is 2-chlorodeoxyadenosine (2-CDA). Rather than directly inhibiting adenosine deaminase, this drug when phosphorylated is resistant to degradation by the enzyme. Phosphoryated derivatives then accumulate in cells with high levels of deoxycytidine kinase activity, including particularly T lymphocytes. This agent is not cell cycle specific, and thus, is particularly effective against indolent neoplasms with low growth fractions.

Several trials have reported benefit in MF/SS with this agent. Investigators at Scripps clinic treated eight patients with a variety of doses of 2-CDA [23]. They observed an overall response rate of 38%, with a median duration of response of three months. A number of patients with non-MF cutaneous T-cell lymphomas were also treated; these patients experienced a 57% response rate. At Northwestern University, we have treated 21 patients with MF/SS [24]. All patients had failed at least one prior therapy. There were three complete responses and three partial responses (major response rate 28%). However, the median duration of response in this heavily pretreated population was only four months. No patient with a large cell variant histology (three patients) responded to therapy. Toxicity in both trials was mild except for myelosuppression, which was most pronounced in patients extensively treated prior to study entry or in patients who received greater numbers of treatment cycles. The myelosuppression may even become most pronounced after discontinuation of the drug.

Two other trials with small numbers of patients with true MM/SS treated with 2-CDA have been published [25,26]. O'Brien and colleagues treated 11

patients with MF/SS as part of a larger trial of T-cell malignancies [25]. They witnessed two responses for an overall response rate of 18%. Betticher et al. summarized their experience with 2-CDA for a number of malignancies; two patients with MF were included in this group [26]. Both patients achieved responses persisting at 3 and 6 months at the time of the report.

Toxicity with 2-CDA is relatively minor. There is little alopecia, nausea, vomiting, or diarrhea associated. Unfortunately, myelosuppression can be fairly dramatic, especially in previously treated patients. In the report by Betticher et al. [26], summarizing toxicity in 51 patients with a variety of malignancies, myelosuppression and infection by bacteria and by opportunistic organisms was commonly observed. In the MF/SS population especially, where skin breakdown and superinfections are a great cause of morbidity, this treatment-related enhanced susceptibility to infection is distressing. It is hoped that perhaps combinations of active agents with 2-CDA might enhance efficacy and allow fewer cycles of therapy to be administered to limit toxicity.

Another enzyme, purine nucleotide phosphorylase (PNP), is also an important regulator of the purine salvage pathway, limiting the intracellular levels of deoxyguanosine triphosphate (dGTP) and deoxyadenosine triphosphate (dATP) levels in human T lymphocytes. If high levels of dGTP and dATP occur, inhibition of ribonucleotide reductase results. If this enzyme could be inhibited, T-cell replication might be reduced. A congenital form of PNP deficiency exists; children exhibit a marked lack of T-cell immune function associated with low T-lymphocyte numbers. The development of clinically useful PNP inhibitors is under way.

Monoclonal antibodies

Monoclonal antibodies have been suggested to be the proverbial 'magic bullet' sought by cancer investigators for decades. These targeted molecules would spare normal tissues while allowing immune system destruction of tumors. MF/SS has been a common disease entity targeted in trials of these modalities because of the easy availability of tumor for observation and biopsy to assess efficacy. To date, most trials have utilized murine monoclonal antibodies because of limitations in the technology required to generate truly 'human' antibodies. Thus, obstacles such as the generation of human antimouse antibodies (HAMAs) have greatly reduced the benefits noted in clinical trials.

The initial trials used unconjugated antibodies that relied on recruitment of immune effector cells to tumor sites for antineoplastic effect. Dillman et al. [27] conducted an early pilot study with T-101, a murine antibody directed against CD-5. Diminution of CD-5-expressing cells was noted in all patients, and 2 of 4 patients with MF experienced partial resolution of several lesions and relief of pruritus. Unfortunately, these effects were short-lived. Subse-

quently, this same group administered high doses of the antibody as an infusion to 10 patients with MF [28]. Forty-percent of the patients again manifested responses, but only slightly more durable than in the earlier trial.

The minimal response witnessed in these early trials led investigators to conjugate toxic moieties to the antibodies to possibly enhance cytotoxicity. Most commonly radioisotopes and toxins have been utilized for this purpose. Rosen et al. conjugated ^{131}I to T-101, utilizing low doses for imaging of lesions and higher doses for therapy. Two partial responses of two months duration were noted, including several dramatic regressions of tumor lesions. Only mild myelosuppression was witnessed. Detection of free ^{131}I was detected due to dehalogenation (breakdown of the immunoconjugate in vivo), and uptake in the thyroid and excretion via the urinary tract was evident. Additionally, all patients developed HAMA. Waldmann [30] and his colleagues at the National Cancer Institute have treated patients with HTLV-I-related cutaneous T-cell lymphomas with a ^{90}Y-labeled anti-Tac monoclonal antibody directed against the interleukin-2 receptor (IL-2R). They observed both partial and complete remissions in these trials.

To avoid toxicity to normal organs, especially the bone marrow, toxins are increasingly being used as the toxic moiety conjugated to an antibody (see Table 2). These molecules share a number of common features: 1) they are heterodimers with two chains, 2) one chain (A-chain) usually imparts cytotoxcity via inhibition of protein synthesis, and 3) the other chain (B-chain) is responsible for binding to mammalian cells and possesses a membrane translocating ability. The toxins most commonly chosen include the plant toxin ricin (either the A-chain alone or with chemical modifications to 'block' non-specific cellular binding), the diphtheria toxin, and *Pseudomonas* exotoxin. An early phase trial of an anti-CD5 antibody conjugated to the ricin A-chain demonstrated several concepts that have been proven through subsequent trials, even with different immunoconjugates [31]. The toxin-labeled antibodies can produce responses. The immunoconjugates have very short half-lives, thus requiring continuous infusions to achieve satisfactory tumor cell exposure times. Immune responses can be brisk, directed against not only the antibody but also the toxin moiety. Antibodies can be either neutralizing or nonneutralizing (which can increase the half-life). Toxicity is related to hepatic scavenging of the immunoconjugate with nonspecific injury (elevated liver

Table 2. Comparison of various targeted toxin modalities for MF/SS

Therapy	No. Patients	Responses	Duration (mos.)
^{131}I-T-101	6	3 (50%)	1–6
H65-RTA	14	4 (29%)	3–8
$DAB_{486}IL$-2	29	6 (21%)	1–49+
$DAB_{389}IL$-2	35	13 (34%)	2.3–22+

function tests, hypoalbuminemia, and a poorly understood capillary leak-like syndrome). At Northwestern University we have tested a blocked-ricin anti-CD6 antibody in patients with relapsed or refractory cutaneous T-cell lymphomas and have witnessed nodal regression, even at low doses.

A final approach utilizing monoclonal antibodies to treat MF/SS relies on the immunogenicity of murine monoclonal antibodies. Foon and his colleagues [32] administered a murine monoclonal antibody 4DC6, an anti-idiotype antibody raised against a monoclonal antibody that targets a highly restricted T-cell antigen expressed on the neoplastic T lymphocytes in T-lymphocyte malignancies, to four patients with MF/SS. A host antibody response to this murine monoclonal antibody should cross-react with the tumor antigen. One of the four patients experienced a major reduction in tumor masses persisting for 11 months. Humoral and cell-mediated responses to the antibody were noted in all patients.

Fusion toxin therapies

Rather than using murine monoclonal antibodies for targeting a cellular antigen, recombinant toxins have been generated by fusion of a plant or bacterial toxin gene to a receptor ligand. The first such fusion toxins to enter clinical trials were DAB_{486}IL-2 and DAB_{389}IL-2, IL-2 receptor (IL-2R)-targeted cytotoxins consisting of nucleotide sequences of the enzymatically active and membrane translocating domains of diphtheria toxin, and the sequence for human interleukin-2 [46]. DAB_{389}IL-2 was a second-generation molecule characterized by a deletion of 97 amino acids from the diphtheria-toxin translocating domain of DAB_{486}IL-2, thus creating a fusion toxin with a more favorable pharmacokinetic profile [33]. Both fusion toxins bind to high-affinity IL-2R, are internalized by receptor-mediated endocytosis, and subsequently inhibit protein synthesis by translocation of the active portion of diphtheria toxin into the cytosol, where it inhibits ADP-ribosylation of elongation factor-2 [34–36]. Activity of both DAB_{486}IL-2 and DAB_{389}IL-2 has been limited to neoplastic lymphocytes or cell lines bearing the high affinity (p55, p75, p64) IL2-R complex, while those cells expressing only a partial form of the receptor (p55 p64, or p75 p64) are insensitive to these toxins.

Although initial phase I/II studies with these fusion toxins included patients with Hodgkin's disease, non-Hodgkin's lymphoma, and MF/SS, the activity of the agents appeared to be most significant in MF/SS (see Table 2). Twenty percent of patients with MF or SS treated in various phase I studies with DAB_{486}IL-2 responded, including one patient with tumor-stage disease who has had a complete response of 36+ months duration [37–40]. A phase II study of DAB_{486}IL-2 was conducted at the NCI and accrued 14 patients with advanced or refractory MF or SS [41]. One patient with extensive plaque-stage disease had a partial response, and two patients with SS had responses that fell just short of the required overall improvement to be considered a partial

response. IL-2R expression was measured in skin and on circulating Sezary cells in the treated patients, and no patient who lacked expression of the high-affinity IL-2R responded to therapy.

A phase I/II clinical trial with DAB_{389}IL-2, the second-generation IL-2 fusion toxin, has been completed for patients with IL-2R-expressing lymphomas, including MF and SS. Immunohistochemical analysis of tumor tissue for IL-2R expression was a prerequisite for entry into this study, and approximately 58% of 75 MF and SS specimens screened were positive for IL-2R [42]. Of 35 treated patients, 13 (34%) have responded [43]. The median duration of response in this heavily pretreated population was 8.0 months. Toxicities have included mild and reversible elevations of hepatic transaminases, mild hypoalbuminemia, fever, and hypersensitivity reactions. Immunologic assessment of the non-Sezary patients demonstrated no change in total numbers of peripheral CD4+, CD8+, or CD4+ CD25+ lymphocyte populations, indicating that there are no secondary immunosuppressive effects associated with this therapy.

Because of these encouraging results with DAB_{389}IL-2 in patients with refractory MF and SS, multicenter phase III studies have been initiated. The goal of these trials will be to verify the above results in less heavily pretreated patients. One study is an open label two-dose study for patients who have failed more than one systemic therapy, and the other will be a double-blind, placebo-controlled, randomized study for patients with stage Ia–III disease who have failed one topical and/or one systemic therapy.

The use of other fusion toxins for targeted therapy of MF and SS is currently under investigation. Recently, two groups (Dalliou et al. and Foss et al.) identified the presence of IL-7 receptors on Sezary cells and growth stimulation of the cells in the presence of IL-7 [44,45]. An IL-7-diphtheria toxin fusion toxin, DAB_{389}IL-7, has been synthesized and has demonstrated cytotoxicity against IL-7 receptor-bearing cells, including Sezary cells, in vitro (F. Foss and J. Murphy, unpublished data). Further clinical development of this fusion toxin is under way.

Thymopentin

Thymopentin (TP5), a synthetic pentapeptide corresponding to the active site of human thymopoietin, has been used in patients with MF and SS in a number of studies and has been associated with dramatic clinical improvement in a subset of patients. In an early study, Przybilla treated eight patients with MF or SS and reported responses in two plaque-stage patients and one patient with SS [46]. The largest clinical experience with thymopentin in MF/SS was reported by Bernengo [47]. In this study, 75% of patients with SS responded to TP-5 at a dose of 50 mg IV three times a week, with 8 of 20 patients attaining a complete response and 7 a partial response. The median time to response after initiation of therapy was 6.1 months (range 2–9), and the median re-

sponse duration for the complete responders was 22 months. Responses were more likely to occur in patients with Sezary counts less than 2600/mm^3, and response was correlated with rise in CD8+ cells and NK cells, suggesting an immunomodulatory activity of this agent. Histopathologic studies of the skin of responding patients demonstrated a loss of epidermotropism, reduction of Langerhans' cells, and decrease in HLA-DR+ keratinocytes.

Another study employing the same dose and scheduling of thymopentin was recently reported from the NCI [48]. In this study of 17 patients with advanced MF and SS, many of whom had failed prior therapies, there was only one partial response: a Sezary patient with weeping ulcers demonstrated clearing of his skin and healing of the ulcers. Six other patients demonstrated a minor response or biological effects, including decrease in pruritus, improvement in skin plaques, or decrease in adenopathy.

The results of these studies suggest that TP-5 may have activity in a subset of patients with MF and SS, most likely in the subset of patients with early-stage disease with low (<2600/mm^3) Sezary counts. The more advanced patients who have failed prior therapy and have higher numbers of circulating Sezary cells, such as those accrued in the NCI study, are not likely to respond. As demonstrated in Bernengo's study, TP-5 has demonstrated immunomodulatory effects that might be useful in combination with other therapies.

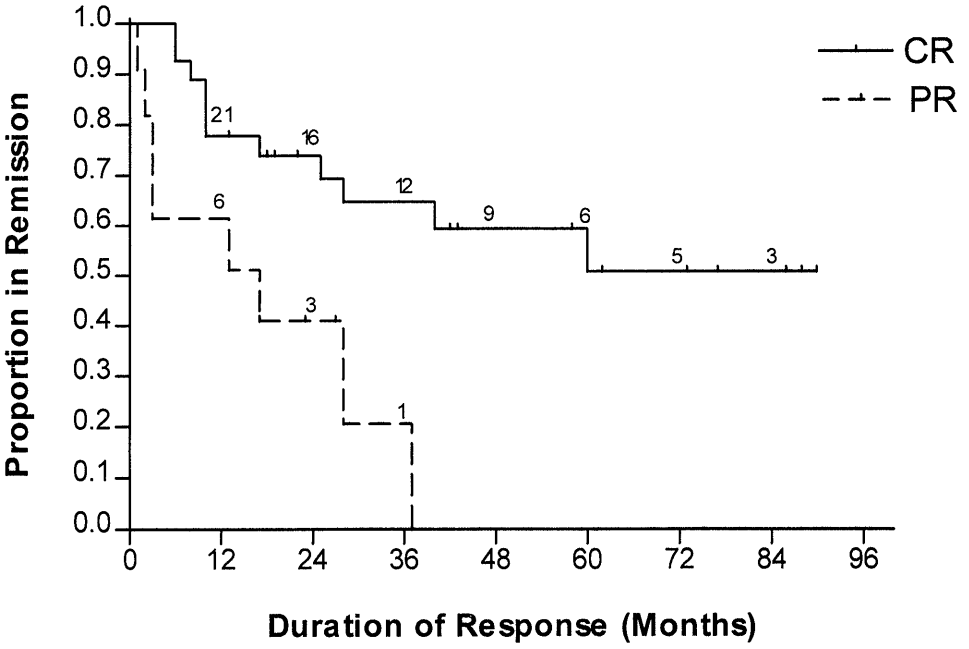

Figure 1. Kaplan–Meier curve demonstrating duration of responses in months by type of objective response. The difference between duration of partial versus complete response is highly significant, with $p = 0.003$.

Combination approaches

The number of active modalities available to treat MF/SS led investigators to develop clinical trials of combinations of modalities in an attempt to enhance duration of response or overall response rates. Many of the clinical trials combined chemotherapy with electron-beam radiation therapy. As noted earlier, a National Cancer Institute trial demonstrated little benefit from this type of combination compared with palliative single agents applied sequentially [1]. More recently, however, a number of clinical trials in other indolent hematologic neoplasms suggested a benefit to either combined chemotherapy plus interferon α or maintenance therapy after chemotherapy with interferon α to prolong remissions [49,50].

Thus, at Northwestern University, we elected to conduct a trial of psoralen with ultraviolet light A (PUVA) topical therapy with interferon α2a for two years to study tolerability and efficacy. We have previously published the details of treatment methodology with results of phase I and II trials [51]. As of 1996, we have completed our studies, with a total of 43 patients treated with the combination. The median age was 55. Thirty-six patients had undergone some prior therapy and 16 patients more than one. The median duration of follow-up for all patient is 52 months. The complete and partial response rates

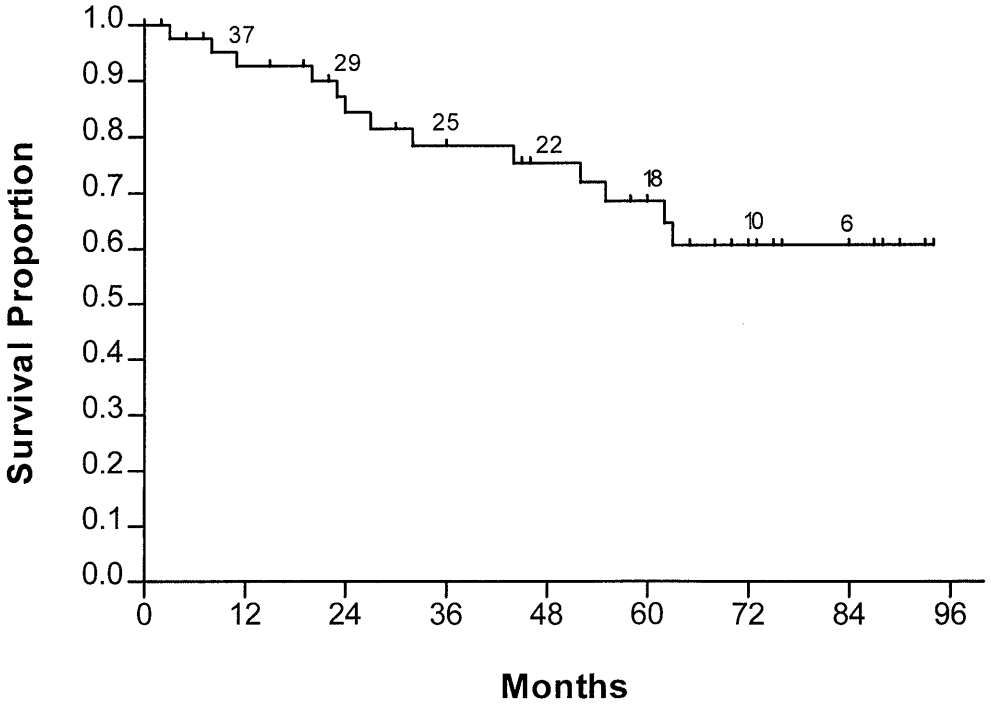

Figure 2. Kaplan–Meier curve demonstrating duration of survival in months for the entire cohort.

were 65% and 26%, respectively. Figure 1 demonstrates the duration of response by type. The median duration of complete response has not yet been reached. The median response duration for partial responders is 17 months. The difference between complete and partial responders is significant, with $p = 0.003$. Figure 2 demonstrates the overall survival of all patients.

These results are dramatically better than observed in trials with PUVA alone or interferon alone. Fully 50% of our patients were stage IIB or worse. Thus, the combination is tolerable, and there is a suggestion of synergistic benefit. In light of these encouraging findings, we have proposed a national randomized cooperative group trial of topical therapies alone compared with topical therapy plus interferon α2a. If significant prolongation of response and survival are noted, changes in the approach to early-stage patients will be warranted.

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10. AIDS-associated lymphoma

Matthew D. Volm and Jamie H. Von Roenn

In 1982, the first report of aggressive B-cell non-Hodgkin's lymphoma (NHL) occurring in homosexual men was published in the medical literature [1]. Three years later, the Center for Disease Control (CDC) added NHL to its list of acquired immune deficiency syndrome (AIDS)-defining illnesses in persons infected with the human immunodeficiency virus (HIV). Today, NHL remains a formidable challenge for physicians caring for HIV-infected persons.

Epidemiology

Epidemiologic data on the incidence of NHL in HIV-infected persons are limited by reporting systems for AIDS that only mandate reporting conditions present at the time AIDS is diagnosed. Conditions that develop after an AIDS diagnosis are not reported, and their impact on the HIV-infected population may therefore be underestimated. Data from the CDC indicate that NHL is a presenting manifestation in 2.9% of newly diagnosed AIDS cases [2]. Similar data from the World Health Organization (WHO) indicate that lymphoma is present at diagnosis in 3.0% of new AIDS cases in Europe [3]. In both the United States and Europe, for reasons that are uncertain, NHL is more often a presenting manifestation of AIDS in hemophiliacs infected through contaminated blood products than in males infected through homosexual contact or in intravenous drug users infected through contaminated needles [2,3]. This may be because male homosexuals and intravenous drug users are at increased risk for earlier development of other AIDS-defining illnesses.

Because NHL often develops after AIDS is diagnosed, the data collected by the CDC and WHO underestimate the risk of an HIV-infected person developing NHL. More meaningful data can be obtained from prospective cohort studies. The National Cancer Institute (NCI) has followed a group of 1065 HIV-seropositive hemophiliacs, whose estimated median time of seroconversion was October 1982. Twelve years later, 12 patients had developed NHL, for an average incidence of 0.15 cases per 100 person-years [4]. The Hemophilia Malignancy Study Group has followed 1295 HIV-

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seropositive hemophiliacs and observed 14 cases of NHL, an incidence of 0.16 cases per 100 person-years (71% as a primary AIDS-defining illness, 29% in patients with a prior AIDS diagnosis) [5]. The Multicenter AIDS Cohort Study (MACS) has followed 5579 gay men since 1984. The cohort includes 2896 HIV-seronegative participants and 2683 participants who were seropositive at the time of enrollment or who subsequently became seropositive. Through 1994, there were 142 cases of NHL reported in the seropositive group (5.3%) and only one case in the seronegative group [6].

The overall risk of an HIV-infected individual developing NHL is uncertain but is clearly greater than the 3% of patients in whom NHL is a presenting manifestation of AIDS. With the advent of effective prophylactic regimens against *Pneumocystis carinii* pneumonia (PCP) and improved treatments for other opportunistic infections, the survival of patients with HIV infection improved substantially in the late 1980s and early 1990s. As more patients have experienced prolonged survival with immunodeficiency, a higher incidence of NHL has been seen [7].

The degree of immune suppression affects the risk and type of lymphoma likely to develop. Primary CNS lymphoma occurs almost exclusively in patients with severe immune deficiency, as reflected by CD4 lymphocyte counts under 50 cells/Ml [8]. Other HIV-associated lymphomas occur across a broader spectrum of immune impairment and show a more modest relationship to the CD4 lymphocyte count [7,8]. Patients who develop small noncleaved cell lymphoma (Burkitt's-like histology) tend to have better preserved immune function than those who develop large cell lymphoma [9–11]. Both the duration and the degree of immune suppression may be important in determining the risk of developing lymphoma.

The effect of antiretroviral drugs on the incidence of NHL has not been well defined. Pluda et al. reported a high incidence of lymphoma in a cohort of 116 patients with advanced HIV infection treated with zidovudine (AZT) or dideoxyinosine (ddI) [12]. Twelve patients developed NHL, yielding an estimated risk of developing NHL within two years of starting antiretroviral therapy of 8.4% (95% confidence interval [CI], 4.1%–16.6%). In this severely immunosuppressed cohort, the CD4 lymphocyte count was inversely related to the risk of developing NHL, and a large proportion of the observed cases were primary CNS lymphoma. In a prospective study of 1030 symptomatic HIV-infected patients with prior AZT therapy, 24 patients developed NHL, and the risk of developing NHL within two years of beginning AZT was 3.2% [13]. A retrospective case–control analysis has shown that exposure to AZT does not confer an increased risk for the development of NHL [14].

The effect that new strategies, such as combination antiretroviral therapy with HIV protease inhibitors, will have on the incidence rate of NHL is difficult to predict. To the extent that the duration of HIV infection is important, lymphoma may become a more common clinical problem as new strategies improve the survival of HIV-infected patients. On the other hand, by

decreasing viral load and slowing the immune dysfunction caused by HIV, new antiretroviral therapies may be effective in preventing some lymphomas, especially those associated with the most severe immune suppression, such as primary CNS lymphoma.

Histology

A large majority of lymphomas in HIV-infected patients are aggressive B-cell malignancies that fall into one of three Working Formulation categories: large noncleaved cell, large cell immunoblastic, and small noncleaved cell. Table 1 shows the breakdown of histologic types of NHL reported in several recent series. The proportion of small noncleaved cell histology is much higher in patients with HIV infection than in the general population, where only approximately 10% of cases are small noncleaved cell lymphomas. Low-grade lymphomas (follicular small cleaved cell and small lymphocytic cell types), which constitute the majority of histologic types seen in the general population, have been reported in HIV-infected persons but are rare and are not AIDS-defining [5,17].

In the Working Formulation, large noncleaved cell lymphomas are considered intermediate grade, whereas the large cell immunoblastic and small noncleaved cell lymphomas are high grade [19]. In the setting of HIV infection, these are all aggressive tumors that frequently present with extranodal disease (see Table 1). The large noncleaved cell and large cell immunoblastic lymphomas are composed of malignant cells with varying degrees of centroblastic or immunoblastic differentiation. The distinction between these two classes is somewhat subjective and not clinically important; for practical purposes, they may be grouped together and referred to as diffuse large cell lymphomas [20]. The small noncleaved cell lymphomas have a distinct histology, with sheets of small, blue lymphoblastic cells punctuated by occasional macrophages, resulting in the classic 'starry sky' appearance.

Table 1. Representative series of HIV-associated lymphomas by histology

	Knowles [15]	Kaplan [16]	Iochim [17]	Raphael [18]
Large non-cleaved cell	25 (28)	17 (20)	40 (40)	35 (31)
Large cell, immunoblastic	25 (28)	36 (43)	31 (31)	33 (29)
Small non-cleaved cell	36 (40)	29 (35)	24 (24)	41 (36)
Other	3 (3)	2 (2)	5 (5)	4 (4)
Extranodal disease present	77 (87)	62 (74)[a]	61 (61)	82 (73)

[a] Patients with predominantly extranodal disease.
Parentheses indicate percentage.

Pathogenesis

Although the precise mechanisms involved in lymphomagenesis in AIDS are unknown, a number of factors are likely important, including Epstein–Barr virus (EBV), human herpes virus-8 (HHV-8), growth stimulation by immunoregulatory cytokines, and the acquisition of specific genetic abnormalities.

Posttransplant lymphoproliferative disorders are an inadequate paradigm

Before the AIDS epidemic, immunosuppression was recognized as a risk factor for the development of NHL in patients with a variety of rare congenital immunodeficiency syndromes and, more commonly, in organ transplant recipients given immunosuppressive drugs to prevent graft rejection [21,22]. As shown in Table 2, lymphomas that develop in organ transplant recipients share a number of characteristics with those that develop in HIV-infected patients. Significant differences in some characteristics of the lymphomas that develop in these two groups suggest that the pathogenetic mechanisms may overlap but are not identical.

Immunosuppressed transplant recipients develop a spectrum of lymphoproliferative disorders ranging from polyclonal proliferations to monoclonal lymphomas. Epstein–Barr virus (EBV) is detectable in virtually all cases, and infection with EBV appears to drive the lymphoproliferative process [23]. Knowles et al. have recently published a useful classification system that divides posttransplant lymphoproliferative disorders into three groups of increasingly aggressive disease [24]:

1. Plasmacytic hyperplasia: these are polyclonal lesions that usually contain multiple forms of EBV and do not demonstrate oncogene or tumor suppressor gene alterations.

Table 2. A comparison of characteristics of posttransplant lymphomas and HIV-associated lymphomas

Posttransplant lymphomas	HIV-associated lymphomas
Extranodal disease common	Extranodal disease common
Primary CNS lymphoma common	Primary CNS lymphoma common
Clinically aggressive	Clinically aggressive
Histologically, large cell lymphomas with varying degrees of heterogeneity and immunoblastic differentiation	Histologically, three types: diffuse large cell, large cell immunoblastic, and small non-cleaved cell (Burkitt's)
Burkitt's-like histology is rare	Burkitt's-like histology common
EBV detectable in almost 100%	EBV detectable in approximately 50%
Both monoclonal and polyclonal tumors are seen	The existence of polyclonal tumors is uncertain; some investigators have reported polyclonal tumors, whereas others have found only monoclonal disease

2. Polymorphic B cell hyperplasia/lymphoma: these are monoclonal lesions that usually contain a single form of EBV but lack oncogene or tumor suppressor gene alterations.
3. Immunoblastic lymphoma: monoclonal lesions that contain a single form of EBV and involve a variety of oncogene and tumor suppressor gene alterations.

Although posttransplant lymphoproliferative disorders are aggressive if untreated, in some cases simply withdrawing the immunosuppression can result in complete disease regression [25]. In other cases, however, even combination chemotherapy fails to control the disease [26].

In contrast to posttransplant lymphoproliferative disorders, EBV is detected in about 50% of systemic HIV-associated lymphomas (excluding primary CNS lymphoma) [27–29]. In addition, small noncleaved cell histology is common in the setting of HIV infection, but is not seen in posttransplant NHL [24]. At a molecular level, this corresponds to the observation that c-myc gene rearrangements are common in HIV-associated lymphomas but are rare in NHL seen in the posttransplant setting.

Aggressive polyclonal lymphoproliferative disorders occur in immunosuppressed transplant recipients, while the existence of 'polyclonal lymphomas' in the setting of HIV infection is controversial. Investigators at the University of California at San Francisco have reported that 35% of the biopsy specimens they studied were polyclonal lesions with no evidence of EBV infection [30]. Hernadier has proposed that a hybrid cell formed by the fusion of an antigen-presenting macrophage with an HIV-infected CD4-positive lymphocyte could be the source of a malignant clone of cells capable of expressing cytokines that stimulate polyclonal B-cell activation and proliferation seen in these lymphomas [31]. Other investigators have identified only monoclonal populations of malignant lymphocytes in their series of HIV-associated lymphomas.

EBV likely plays a role in some, but not all, HIV-associated lymphomas

Although the association between EBV and lymphoma is not as strong in HIV-infected patients as in transplant recipients, EBV likely plays a role in the pathogenesis of some HIV-related lymphomas. EBV infection results in the immortalization and proliferation of infected B cells. In an immunocompetent host, a T-cell response contains the EBV infection, resulting in a latent carrier state [32]. Without an adequate T-cell response, however, EBV-driven B-cell proliferation continues unchecked. The unsuppressed expansion of B cells increases the likelihood of chromosomal mutations capable of giving rise to a malignant clone. Mutations that result in the activation of the c-myc oncogene are a hallmark of both endemic and sporadic Burkitt's small noncleaved cell lymphoma [32]. Clonal EBV is present in 100% of endemic Burkitt's lymphoma and in 10%–20% of sporadic cases [33]. It is hypothesized that falciparum malaria infection causes the impairment of

T-cell immunity in endemic Burkitt's lymphoma and explains its geographic distribution [34].

The progressive loss of T-cell function that occurs in HIV-infected patients suggests that they are at risk for EBV-driven lymphoproliferative disorders. The presence of detectable EBV in benign enlarged lymph nodes in these patients is associated with an increased risk of lymphoma [35]. As noted above, EBV is detectable in about 50% of systemic lymphomas associated with HIV. In primary CNS lymphoma, which occurs almost exclusively in patients with severe immunosuppression (CD4 lymphocyte count less than 50 cells/μL), EBV is present in 100% of cases [36,37]. Although the precise role of EBV in primary CNS lymphoma is not known, as discussed below, the expression of EBV latent membrane protein 1 (LMP1) may act to prevent apoptosis in malignant cells [38].

In addition to causing the loss of T-cell function that may permit the unchecked proliferation of EBV-infected lymphocytes, there is laboratory evidence that HIV can infect B cells previously infected with EBV [39]. Coinfection with EBV and HIV in vitro results in frequent c-myc oncogene expression and in the expression of EBV proteins that may contribute to malignant transformation [39].

The precise nature of the relationship between EBV and systemic lymphoma in the setting of HIV infection remains uncertain. Based on the historic association between EBV and endemic Burkitt's lymphoma, it might be expected that EBV would be frequently found in the small noncleaved cell lymphomas associated with HIV. In fact, most series report that EBV is more often detected in HIV-associated lymphomas of large cell histology [27,28]. The lack of a consistent relationship between EBV and small noncleaved histology suggests that HIV-related lymphomas of small noncleaved cell histology may be pathogenetically more similar to sporadic than to endemic Burkitt's lymphoma.

The recently discovered Kaposi's sarcoma herpes virus (KSHV) is associated with body cavity-based lymphomas in patients with HIV infection

The recently discovered Kaposi's sarcoma herpes virus (KSHV), or human herpes virus 8 (HHV-8), is associated with an unusual group of HIV-related lymphomas that present with pleural, peritoneal, and pericardial involvement and thus are body cavity based [40]. HHV-8 was discovered in Kaposi's sarcoma lesions after epidemiologic data suggested that Kaposi's sarcoma is caused by an infectious agent [41]. Subsequently, biopsy specimens from lymphomas in both HIV- and non-HIV-infected patients were analyzed. Evidence of HHV-8 DNA sequences was found in eight HIV-related, body-cavity lymphomas; none of the other specimens showed evidence of HHV-8 [40]. The role of HHV-8 in the pathogenesis of body cavity-based lymphoma is under investigation.

HIV infection causes both chronic B-cell stimulation and loss of regulatory T-cell function

Initial infection with HIV results in the widespread dissemination of virus and high levels of viremia. Within weeks to months after the initial infection, HIV-specific antibodies develop and a decline in viremia is seen. This immunity, however, fails to suppress viral replication, particularly in lymphoid organs, which act as reservoirs of HIV during the latency period that precedes the development of clinical AIDS [42]. During this period, HIV infection causes two processes that may predispose patients to the development of lymphoma: chronic, nonspecific B-cell stimulation and the loss of normal T-cell function.

The immune system normally reacts to a foreign antigen by orchestrating a response that includes the clonal expansion of B cells that produce high-affinity antibodies against the antigen and the activation of T cells involved in both eliminating foreign antigen and regulating the B-cell response. In HIV infection, in addition to the normal specific response to HIV antigens, there is a nonspecific immune response that is marked by polyclonal activation and expansion of B cells [43–45]. This is reflected clinically in the persistent generalized lymphadenopathy, polyclonal hypergammaglobulinemia, and high incidence of autoimmune phenomena seen early in the course of HIV infection.

The chronic B-cell stimulation in HIV infection is a source of proliferating lymphocytes that could potentially acquire the genetic abnormalities that underlie malignant transformation [46]. Evidence that chronic immune stimulation confers an increased risk of developing lymphoma is seen in a variety of autoimmune disorders such as Sjögren's syndrome, systemic lupus erythematosus, and sarcoidosis [22].

The cause of the B-lymphocyte activation in HIV infection is not known, but several factors may be important:

1. HIV may directly cause polyclonal activation of B lymphocytes. Peripheral-blood B cells from uninfected individuals can be induced to proliferate in vitro when cultured in the presence of HIV [45]. The mechanism by which HIV stimulates B cells is unknown. Recent studies have focused on the role of HIV envelope glycoproteins (gp 120, gp 160) acting either as superantigens capable of stimulating a wide variety of B cells or as stimulants to B-cell secretion of growth-promoting cytokines, such as interleukin 6 (IL-6) [47–49].
2. HIV may induce autoimmune responses through molecular mimicry. A number of HIV proteins have regions homologous to important components of the human immune system. Gp 120 and gp 41 have regions homologous to MHC class I and II molecules, variable and constant chains of the T-cell receptor, and multiple domains of immunoglobulin molecules [50]. Hernadier et al. at the University of California at San Francisco have

reported a high incidence of CD5 positivity in a series of lymphomas they studied. CD5 is an antigen associated with autoreactive B lymphocytes [31]. Its presence would suggest that autoreactivity is a potential mechanism by which predominant clones could be selected in the setting of polyclonal B-cell activation and eventually evolve into malignancy.

3. Depressed T-cell regulatory activity is unlikely to play an important role in the B-cell stimulation seen early in the course of HIV infection [46]. The clinical signs and symptoms of chronic B-cell activation do not correlate with T-lymphocyte levels during the early stage of HIV infection, and in vitro the presence of noninfected T cells does not effect the activation of B cells [51]. The loss of T-cell function may be most important in primary CNS lymphoma, which occurs late in the course of HIV infection and is associated with EBV-driven B-cell proliferation.

Although much remains to be learned about the effects of HIV infection on B lymphocytes, it is likely that the chronic B-cell stimulation that accompanies HIV infection plays an important role in the lymphomagenesis of some HIV-associated lymphomas.

Endogenous cytokines may be important growth factors in AIDS-related lymphoma

Increased cytokine production in response to HIV infection is likely involved in both the nonmalignant activation and proliferation of B lymphocytes seen in patients with HIV-associated lymphadenopathy and in the development of malignant lymphoma. While increased serum levels of a variety of cytokines have been reported in the setting of HIV infection (e.g., interferon-alpha, tumor necrosis factor), interleukin-6 (IL-6) is most often postulated to be important in AIDS lymphomagenesis [52,53].

IL-6 is an important factor in acute phase reactions and has pleiotropic effects on several cell lines. It is produced by monocytes, B cells, fibroblasts, endothelial cells, astrocytes, and microglial cells; it activates resting B cells and causes them to express immunoglobulin genes and to differentiate into plasma cells. HIV infection induces the expression of IL-6 [54–56], and serum IL-6 levels are positively correlated with the risk of developing lymphoma in patients infected with HIV [57]. Emilie et al. have recently examined the production of IL-6 by malignant B cells taken from 24 patients with high-grade immunoblastic or small noncleaved cell lymphomas, 18 of whom were infected with HIV [58]. They found IL-6 gene expression in all the samples analyzed. There was a strong positive correlation between the level of IL-6 production and immunoblastic histology. Immunostaining localized the IL-6 production predominantly to nonmalignant macrophages and endothelial cells, suggesting the possibility of paracrine growth stimulation of the malignant immunoblasts. Levine et al. followed serum IL-6 levels in 28 patients with HIV-associated lymphoma treated concomitantly with m-BACOD chemotherapy and zalcitabine (ddC). Elevated IL-6 levels correlated with the presence of consti-

tutional 'B' symptoms at diagnosis and, over time, with serum HIV p24 antigen levels, LDH, and response to therapy [59].

The molecular genetics of HIV-associated lymphomas are being investigated

The normal differentiation and clonal proliferation of B lymphocytes in response to an antigen are marked by rearrangements of gene sequences that encode immunoglobulin genes. It is the large number of potential combinations that allows the immune system to respond to a wide variety of encountered antigens. Although the molecular events that underlie lymphomagenesis are incompletely understood, a recurrent theme is the translocation of a normal growth-promoting gene (a proto-oncogene) to an immunoglobulin gene region involved in the differentiation and clonal expansion of B cells. These translocations result in the inappropriate expression of the growth-promoting gene and thus contribute to the malignant transformation of the cell. An example is the t(8,14) translocation, which causes the expression of the c-myc oncogene in Burkitt's and other lymphomas. The precise function of the c-myc gene is unknown; its product is a nuclear protein involved in the regulation of other genes affecting cell proliferation and differentiation. In addition to the classic t(8;14) translocation, other translocations involving immunoglobulin genes, such as t(8;22) and t(2;8), can lead to c-myc activation. C-myc rearrangement is present in virtually all Burkitt's lymphomas.

Some investigators have found c-myc rearrangements in all or nearly all small noncleaved cell lymphomas associated with HIV infection [28,60], suggesting that a common genetic mechanism underlies their morphologic similarity to classic Burkitt's lymphoma. Other investigators, however, have found c-myc rearrangements in only half of small noncleaved cell lymphomas [27,29]. C-myc rearrangements are detected less frequently in diffuse large cell lymphoma and have not been detected in primary CNS lymphomas [27–29,60].

Other oncogenes that may be involved in lymphomagenesis in AIDS have not been studied as extensively as c-myc. Rearrangements of bcl-2, an oncogene often involved in low-grade follicular center cell lymphomas, have not been identified in HIV-associated lymphomas [61]. Activation of the ras oncogene has been associated with multiple myeloma and acute lymphoblastic lymphoma, but not other lymphoid malignancies in the past. A recent study demonstrated ras activation in 15% of HIV-associated lymphomas [27]. The same investigators found inactivation of the p53 tumor suppressor gene in 10 of 16 small noncleaved cell lymphomas but in none of the diffuse large cell lymphomas examined.

Recently, a new proto-oncogene associated with 30%–40% of diffuse large cell lymphomas in non-HIV-infected patients has been identified [62]. Located on chromosome 3, the BCL-6 gene encodes a protein that contains multiple zinc finger repeats; it is expressed in mature resting B cells in germinal centers but not in activated immunoblasts or plasma cells. It is likely that BCL-6 is

downregulated when resting B cells are stimulated by antigen to proliferate and differentiate into antibody-secreting plasma cells. In diffuse large cell lymphoma in the general population, BCL-6 mutations are associated with extranodal involvement, lack of bone marrow involvement and a favorable prognosis. Gaidano et al. recently detected rearrangements of BCL-6 in 20% of HIV-associated diffuse large cell lymphomas; no BCL-6 rearrangements were seen in biopsy specimens with small noncleaved cell histology [63].

Presentation, staging, and prognostic factors

Extranodal involvement is a hallmark of HIV-associated lymphoma. Table 3 shows the most common extranodal sites seen in several large series. Although the CNS, bone marrow, liver, and GI tract are the most frequent extranodal sites, lymphoma involving other areas is common in the setting of HIV infection. HIV-related NHL has been reported in almost every conceivable anatomic location, including the heart, pericardium, buccal mucosa, gingiva, eye, rectum, and anus [53]. At diagnosis, 80%–90% of patients have constitutional symptoms, including weight loss, fevers, night sweats, and fatigue [64].

The diagnosis of lymphoma in a patient with HIV infection is often delayed because the symptoms mimic those of an opportunistic infection or because unusual extranodal presentations are overlooked. The radiographic appearance of HIV-related lymphoma may also mimic opportunistic infections; pleural effusions, interstitial and alveolar infiltrates, and parenchymal lung nodules are not uncommon manifestations of HIV-related lymphoma in the chest [65]. A diagnosis of lymphoma can be made by fine needle aspiration or excisional biopsy. Fine needle aspiration requires a cytologist with expertise in diagnosing lymphoma and may be nondiagnostic due to sampling error. Tissue samples should be sent for appropriate stains and cultures to look for mycobacterial and fungal infection. Flow cytometry analysis of the specimen may be useful in questionable cases by identifying B-cell surface antigens and demonstrating monoclonal kappa or lambda light chain restriction.

The evaluation of a new patient with HIV-associated NHL requires careful

Table 3. Frequent sites of extranodal disease

	Knowles [15]	Kaplan [16]	Raphael [18]
Total patients	89	82	113
Patients with extranodal disease at presentation	77	72	82
CNS	19 (includes 8 primary CNS)	17 (includes 4 primary CNS)	22 (17 brain, 5 meninges)
Bone marrow	19	26	17
Liver	14	22	3
GI tract	24	3	18

attention in order to rule out concurrent opportunistic infections and to identify all sites of disease. The evaluation should routinely include CT scans of the chest, abdomen, and pelvis; bilateral bone marrow biopsies; and a lumbar puncture. Laboratory studies should include a complete blood count, chemistries, CD4-positive lymphocyte count, and an evaluation of HIV viral burden. Other tests should be ordered as dictated by the patient's symptoms. The Ann Arbor staging system for lymphoma was developed for Hodgkin's disease and is based on the usual pattern of Hodgkin's disease progression to contiguous nodal groups. This staging system is of limited usefulness in AIDS-associated NHL, since the Ann Arbor stage does not alter therapy and is not of prognostic significance.

The prognosis for HIV-infected patients with NHL is poor, although prolonged survival is possible for a small percentage of patients. For aggressive non-Hodgkin's lymphomas in the general population, the International Non-Hodgkin's Lymphoma Risk Factor Project has identified five risk factors that predict survival: patient age, performance status, serum lactate dehydrogenase (LDH), stage of disease, and the number of extranodal sites [66]. In patients with HIV infection, in whom widespread disease is common, the stage of disease and the number of extranodal sites are not prognostically significant [67]; histologic type also does not affect prognosis. The CD4 lymphocyte count, reflecting the patient's underlying immune status, is the best predictor of survival [67–69]. In a series of 84 patients treated at the San Francisco General Hospital, median survival was 24 months for patients with a CD4 lymphocyte count of greater than 100 cells/mm^3, whereas for patients with fewer than 100 cells/mm^3, median survival was only four months [68]. Other variables that may affect prognosis are age, performance status, LDH, a prior history of CDC-defined AIDS, and the presence or absence of extranodal disease. Patients with primary CNS disease have a particularly poor prognosis, with median survival reported in the range of 3–5 months [70–72]. Leptomeningeal involvement secondary to primary systemic disease does not carry the same grave prognosis as primary CNS disease and may have minimal impact on survival [73].

Treatment

The treatment of AIDS-related NHL has evolved against a background of significant changes both in the treatment of NHL in the general population and in the treatment of HIV infection. During the past 10 years, prophylaxis against a variety of opportunistic infections and the use of antiretroviral drugs have resulted in a improved quality of life and longer survival for patients with HIV infection. In 1993, the results of two randomized trials resulted in a shift in the treatment of aggressive NHL in non-HIV-infected patients from combination chemotherapy regimens emphasizing high-dose intensity and exposure to a large number of drugs early in the course of treatment to standard CHOP

chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) [74,75].

The role of dose intensity in HIV-associated lymphoma

The first published evidence that increasing dose intensity could be counterproductive in the setting of HIV-associated NHL was provided by Gill et al., who in 1987 reported the results of two consecutive series of patients [76]. The first 13 patients were treated with M-BACOD, a standard second-generation combination-chemotherapy regimen, and the second nine patients were treated with a more dose-intensive regimen that included high-dose cytarabine, high-dose methotrexate, L-asparaginase, cyclophosphamide, vincristine, and prednisone. There was no statistically significant difference in complete response rates between the two groups (54% for group 1 and 33% for group 2), but the second group had poorer survival due to opportunistic infections (one-year event-free survival was 31% for group 1 and 11% for group 2). This experience led the AIDS Clinical Trials Group (ACTG) to investigate lower doses of myelosuppressive drugs for patients with AIDS-associated NHL [73]. Table 4 shows the reduced dose m-BACOD regimen used in ACTG 008. Zidovudine (AZT) anti-retroviral therapy was given to patients after the completion of chemotherapy. Sixteen patients (45.7%) achieved a complete response; median survival for all evaluable patients was 6.5 months, but for patients achieving a complete response, median survival was 15 months [73]. CNS prophylaxis was given to all patients, and no patient experienced a relapse involving the CNS.

While ACTG 008 was being conducted in the U.S., the French–Italian Study group investigated the strategy of selecting treatment based on the patient's performance status and history of prior opportunistic infections

Table 4. The low-dose m-BACOD regimen of ACTG 008 and ACTG 142

Day 1
Cyclophosphamide 300 mg/m^2 IV
Doxorubicin 25 mg/m^2 IV
Vincristine 1.4 mg/m^2 IV
Bleomycin 4 u/m^2
Day 1–5
Dexamethasone 3 mg/m^2 PO
Day 15
Methotrexate 500 mg/m^2 IV[a]
Folinic acid rescue, 25 mg PO q 6 h × 4 beginning 6 hours after methotrexate
Day 1, 8, 15, 28
Cytarabine 50 mg intrathecally as CNS prophylaxis

[a] In the randomized trial ACTG 142, methotrexate was given at 200 mg/m^2 without folinic acid rescue.

[77,78]. Patients with a World Health Organization (WHO) performance status greater than or equal to 3 or a history of prior opportunistic infection were considered to be at high risk for complications of therapy and progression of AIDS. They were treated with a 50% reduced-dose chemotherapy regimen that included cyclophosphamide, doxorubicin, teniposide, prednisone, vincristine, and bleomycin; AZT was given concurrently. Patients with a performance status less than or equal to 2 and no history of opportunistic infection were considered to be better candidates for intensive therapy and were treated with a modified LNH84 regimen (doxorubicin, cyclophosphamide, bleomycin, and prednisolone for three cycles, followed by consolidation with high-dose methotrexate, ifosfamide, etoposide, asparaginase, and cytarabine); AZT was given following chemotherapy. Outcome was poor in both groups: in the high-risk, low-dose group, median survival was 3.5 months; in the low-risk, intensive-therapy group, median survival was nine months [77,78].

The role of dose intensity in HIV-related NHL has been evaluated in a randomized, prospective trial, ACTG 142, a multicenter study comparing low-dose m-BACOD to standard-dose M-BACOD with colony stimulating factor support [79]. As shown in Table 5, there was no difference in the complete response rate or median survival between the two groups, but patients in the low-dose group had significantly less therapy-related hematologic toxicity than those in the high-dose group, in spite of the use of recombinant granulocyte–macrophage colony stimulating factor (GM-CSF) in the high-dose group [79]. Although not statistically significant, a trend was observed in the causes of death: patients who received low-dose m-BACOD tended to die more often of lymphoma, whereas patients who received standard-dose chemotherapy tended to die more often of other complications of HIV infection. Grade IV neutropenia was significantly more common among patients with low CD4 lymphocyte counts: 70% of patients with a CD4 lymphocyte count less than 100 cells/mm^3 experienced grade IV neutropenia, compared to 39% of patients with a CD4 lymphocyte count greater than 100 cells/mm^3.

During the time ACTG 142 was accruing patients, new information became available regarding the role of second-generation combination chemotherapy

Table 5. Results of ACTG 142

	Low-dose m-BACOD	Standard-dose M-BACOD
Complete responses	39 (41%, $N = 94$)[a]	42 (52%, $N = 81$)[a]
Median survival	35 weeks[a]	31 weeks[a]
Deaths due to lymphoma only	41 (59%, $N = 70$)[b]	27 (38%, $N = 70$)[b]
Deaths due to HIV only	18 (26%, $N = 70$)[a]	21 (30%, $N = 70$)[a]
Deaths due to HIV and lymphoma	8 (11%, $N = 70$)[a]	13 (19%, $N = 70$)[a]
Grade 4 neutropenia	49 (50%, $N = 98$)[c]	65 (65%, $N = 94$)[c]

[a] p not significant.
[b] $p = 0.06$.
[c] $p = 0.007$.

regimens for NHL in patients without HIV infection. Two multi-institution randomized trials demonstrated that CHOP chemotherapy was equivalent to M-BACOD in terms of response rates and survival and caused less treatment-related toxicity than M-BACOD in patients not infected with HIV [74,75]. Standard therapy for HIV-associated NHL is now either reduced-dose CHOP or the reduced-dose m-BACOD regimen of ACTG 142.

Because patients with HIV infection tolerate myelosuppressive therapy poorly, investigators are focusing efforts on less toxic therapies. The Canadian HIV Trials Network evaluated low-dose therapy with etoposide, vincristine, prednisone, mitoxantrone, and cyclophosphamide [80]. Colony stimulating factor was not given. Median survival for the group (n = 29, median CD4 lymphocyte count 55 cells/mm^3) was 4.5 months, and febrile neutropenia complicated 21 of 91 cycles of chemotherapy. As suggested by this experience, dose reduction is an intrinsically limited strategy for improving the tolerability of chemotherapy.

Recent advances in anti-retroviral therapy, particularly the advent of highly active combination drug regimes, may enhance the ability of patients with HIV infection to tolerate chemotherapy.

Myelosuppression and the use of colony stimulating factors

Patients with HIV infection often have poor hematologic reserve due to the marrow-suppressive effects of both HIV itself and the drugs used to treat HIV and its infectious complications. Patients with advanced HIV infection often experience severe neutropenia when given even low doses of myelosuppressive drugs. The use of myeloid colony stimulating factors has therefore become routine in patients with HIV infection receiving chemotherapy for NHL. Because HIV infects cells of monocyte and macrophage lineage, the possibility of increased HIV replication with colony stimulating factors was initially a concern. Investigators at the University of California at San Francisco therefore evaluated the use of GM-CSF in HIV-associated lymphoma in a randomized trial. Patients were treated with CHOP chemotherapy and randomized to receive prophylactic GM-CSF following each cycle of therapy or no additional treatment [81]. Response rates and survival were equivalent in both groups (complete response seen in 67% of control group and 70% of GM-CSF group; median survival 9.0 months for the control group and 11 months for GM-CSF group). Neutropenic fever was substantially less frequent in the GM-CSF group: 74% of chemotherapy cycles on the control arm were complicated by neutropenic fever, compared with 34% of cycles on the GM-CSF arm. The investigators found a 243% increase in HIV p24 antigenemia after three cycles of therapy in the GM-CSF group but not in the control group, suggesting GM-CSF stimulation of HIV replication. This effect was transient, however, and at four weeks after the completion of treatment, p24 antigen levels had returned to 120% of their pretreatment levels in the GM-CSF group.

In light of the results of this trial, stimulation of HIV replication by colony stimulating factors is not a major concern, particularly when compared to the benefit of reduced febrile neutropenia. Recombinant granulocyte colony stimulating factor (G-CSF) became available after GM-CSF was evaluated. Since it has less effect on cells of macrophage and monocyte lineage, it is considered less likely than GM-CSF to stimulate HIV replication. Additionally, the use of nonmyelosuppressive antiretroviral agents during treatment for NHL limits the concerns about growth-factor-induced upregulation of HIV.

Secondary CNS disease: prophylaxis and treatment

CNS prophylaxis for patients with AIDS-associated NHL is controversial. In the 22 patients described by Gill et al., three were found to have CNS disease at the time of diagnosis, and 8 of the remaining 19 patients progressed in the CNS in spite of systemic therapy [76]. This high rate of CNS relapse, as well as reports of frequent CNS involvement at autopsy, led to the use of CNS prophylaxis with intrathecal cytarabine in all patients treated with low-dose m-BACOD on ACTG 008 [73]. None of these patients relapsed with CNS involvement, and some clinicians routinely give CNS prophylaxis to all patients with HIV-associated NHL. Other clinicians believe that the high rate of CNS relapse in the series reported by Gill was a result of higher than usual rates of small noncleaved cell histology and bone marrow involvement and recommend CNS prophylaxis only when one of these risk factors is present.

All patients must have a lumbar puncture as part of their initial staging evaluation. Patients with positive CSF cytology require aggressive intrathecal therapy, almost always via an Ommaya reservoir. Although there is no defined standard regimen, a reasonable approach is to administer cytarabine 50 mg in 5 cc of preservative-free normal saline three times per week until the CSF is clear. After the CSF is clear, treatment can be given weekly for four weeks and then every four weeks to complete a full year of therapy. Intrathecal methotrexate is also effective, but is more frequently associated with meningeal irritation. Sites of bulky meningeal disease are best treated with radiation, and in some centers all patients with meningeal disease routinely receive neural axis radiation.

Concurrent chemotherapy and antiretroviral therapy

Administering antiretroviral therapy concurrently with chemotherapy is desirable, since many patients with AIDS-associated NHL die from complications of progressive HIV infection. Chemotherapy may cause lymphopenia and further depletion of CD4-positive lymphocytes. As long as zidovudine (AZT) was the only antiretroviral drug available, concurrent antiretroviral therapy was difficult, since the myelosuppression caused by AZT overlaps with the myelosuppression of chemotherapy. Since the early 1990s, however, other

antiretroviral drugs have been available. There is evidence that the nucleoside analogue didanosine (ddI) may ameliorate the myelosuppression of cytotoxic therapy [82–84]. A randomized phase III trial of chemotherapy (mitoxantrone, etoposide, cyclophosphamide, bleomycin, vincristine, and methylprednisolone) and concomitant ddI versus chemotherapy alone showed no statistically significant difference in survival but did show a trend in favor of the ddI arm [84]. The safety of administering the new reverse transcriptase inhibitors and HIV protease inhibitors now on the market concurrently with cytotoxic chemotherapy has not been firmly established, but it is likely that these drugs will be included in future clinical trials. In the meantime, most clinicians favor administering highly active antiretroviral therapy concurrently with chemotherapy. Because of myelosuppression. AZT is avoided. It is hoped that highly active antiretroviral therapy will improve the tolerability of chemotherapy for patients with HIV.

Second-line therapy for refractory or relapsed disease

There is no established second-line therapy for patients with refractory or relapsed HIV-related NHL, and there are limited data to guide clinicians in treating these patients. Patients not infected with HIV often receive salvage therapy with any one of several published second-line regimens. These regimens are generally not curative, but patients who respond are candidates for high-dose therapy with autologous stem cell support, a strategy that may be curative in up to 40% of patients. For patients with HIV infection, salvage therapy for relapsed or refractory disease is usually poorly tolerated, and dose-intensive strategies designed to overcome drug resistance are not practical. The Italian Cooperative Group on AIDS and Tumors has reported their experience treating 19 patients with relapsed or refractory disease with etoposide (VP-16), mitoxantrone, and prednimustine (VMP) [85]. They reported a 37% response rate, with the majority of responses in patients with relapsed disease. Median survival was seven months in patients with relapsed disease and two months in patients with refractory disease.

Levine et al. treated 31 patients with relapsed or refractory HIV-associated lymphoma with mitoguazone (methl-glyoxal-bis guanylhydrazone [MGBG]), a cytotoxic agent that interferes with the synthesis of polyamines, believed important in the stabilization of DNA [86]. MGBG was evaluated because of its minimal hematologic toxicity and good CNS penetration. Of 25 patients evaluable for response, two complete responses (8%) and five partial responses (20%) were observed. Toxicity was mild, and improved performance status was seen in 33% of patients.

New approaches to the treatment of HIV-associated lymphomas

New approaches to the treatment of HIV-associated lymphoma are being actively investigated. Sparano et al. have reported a 62% complete response

rate and a median survival of 18 months for a group of 30 patients treated with cyclophosphamide, doxorubicin, and etoposide (CDE) given as a 96-hour infusion [87,88]. This approach is presently being evaluated by the Eastern Cooperative Oncology Group (ECOG).

Biologic therapies, such as monoclonal antibodies and cytokines, for HIV-related NHL are theoretically attractive for several reasons: they may be less myelosuppressive and better tolerated than conventional chemotherapy; they may have immunostimulating properties that favorably affect the underlying immunosuppression; and they may represent a form of non-cross-resistant therapy capable of killing malignant cells that are resistant to conventional drugs.

Several trials of biologic agents have been conducted in patients with AIDS-related NHL. Tupule et al. treated nine patients with heavily pretreated relapsed or refractory disease with B4bR, a toxin-linked antibody against the CD19 antigen present on normal B cells and B-cell lymphomas [89]. Toxicity was mild and two patients responded. Emilie et al. treated 11 patients with AIDS-related NHL (five patients with relapsed disease and six newly diagnosed) with an anti-IL-6 antibody BE-8 [90]. They observed one partial response, stabilization of disease in five patients, and disease progression on therapy in five patients. Interestingly, they also observed rapid and complete resolution of fever and night sweats in four patients who presented with these symptoms, as well as substantial weight gain in all eight patients who completed the 21-day infusion. They concluded that anti-IL-6 therapy has a modest effect on the growth of lymphoma but is capable of abrogating clinical 'B' symptoms. Bernstein et al. have investigated the use of interleukin 2 (IL-2), a cytokine with immune-stimulating properties, on 10 patients with HIV-related malignancies, five with lymphoma and five with Kaposi's sarcoma [91]. They observed an increase in natural killer cell activity and an increase in eosinophils. No patient developed an opportunistic infection during 50 months of therapy.

Biologic therapy with a humanized monoclonal antibody targeting CD20, a B cell surface antigen associated with calcium channels and cell activation events, has demonstrated efficacy in low grade and follicular lymphomas in the general population [92]. Because of its lack of myelotoxicity, anti-CD20 therapy may be especially useful in patients with HIV infection and is currently being investigated in combination with CHOP chemotherapy in patients with HIV-related NHL.

A role for biologic agents in HIV-related NHL is not yet established. Many investigators believe that these agents may be most useful when disease burden is low and therefore that they may have a role in consolidation or maintenance therapy following conventional cytoreductive chemotherapy. In addition, Khatri et al. have suggested that biologic therapy may have a role in preventing HIV-related NHL [93]. In laboratory models, they have shown that low dose IL-2 inhibits the development of B cell proliferative disease associated with EBV.

Primary CNS lymphoma

In the general population, primary CNS lymphoma is a rare disease, accounting for only 0.5%–1.2% of primary brain tumors and approximately 2% of non-Hodgkin's lymphoma [70,94]. In patients with HIV infection, however, primary CNS lymphoma is the second most common mass lesion in the brain, following toxoplasmosis, and accounts for 10%–20% of lymphomas [3]. The association between immunodeficiency and primary CNS lymphoma was recognized before the onset of AIDS in patients with congenital immunodeficiency syndromes and in organ transplant recipients given immunosuppressive drugs to prevent graft rejection [21,22]. In the setting of HIV infection, primary CNS lymphoma is associated with severe immunosuppression, as reflected by CD4 lymphocyte counts under 50 cells/mm^3 in most patients [8].

The high frequency with which EBV is detectable in HIV-associated primary CNS lymphoma suggests a unique pathogenesis. Using an in situ hybridization technique, Bashir et al. identified EBV in each of four specimens obtained from immunocompromised patients (three patients with AIDS and one allograft recipient) but in none of the specimens obtained from four immunocompetent patients with primary CNS lymphoma [95]. MacMahon et al. found evidence of EBV in all of the 21 specimens from HIV seropositive patients they studied [96]. Latent membrane protein 1 (LMP1) is a product of EBV that has been reported in vitro to transactivate the bcl-2 gene, a proto-oncogene that prevents apoptosis [97,98]. Bcl-2 is expressed in follicular lymphomas and in B and T cells at various stages in their development [99,100]. Recently, Camilleri-Broet et al. have examined the expression of LMP 1 and bcl-2 in 68 AIDS-associated lymphoma specimens. They found expression of both LMP1 and bcl-2 in 10 of 11 primary CNS lymphomas [101]. Of 53 cases of systemic lymphoma, LMP1 was detected in 21 and bcl-2 in only three. The investigators conclude that LMP1-induced bcl-2 expression may be important in the pathogenesis of HIV-associated lymphoma arising in the CNS but not in other locations.

Diagnosing primary CNS lymphoma in patients with HIV infection is difficult. The appearance of the lesions on CT or MRI scan is not specific and cannot be distinguished from toxoplasmosis. Toxoplasmosis is the most frequent cause of focal CNS lesions in patients with AIDS (50%–70%), followed by primary CNS lymphoma (20%–30%) and progressive multifocal leukoencephalopathy (PML) (10%–20%). Processes such as tuberculous, cryptococcal infection and bacterial abscesses are less common [102]. MRI scan is more sensitive than CT in the evaluation of HIV-infected patients with neurologic symptoms and is the imaging study of choice [103].

Two new techniques, which rely on advanced technologies not routinely available at all centers, appear to be helpful in diagnosing primary CNS lymphoma. The first is the use of the polymerase chain reaction (PCR) to detect the presence of EBV in the cerebral spinal fluid (CSF), which predicts for CNS lymphoma. PCR for EBV appears to have moderate sensitivity and

high specificity for lymphoma; in one study the sensitivity was 80% and the specificity was 100% [104]; in a smaller trial sensitivity was 64% and specificity was 82% [105]. Assays for EBV in CSF are instrinsicially limited by the potential risks of obtaining spinal fluid in patients with parenchymal brain lesions and may not be possible for many patients.

In addition to PCR for EBV in CSF, positive imaging on thallium SPECT scan is useful in distinguishing lymphoma from other causes of parenchymal CNS lesion in the setting of HIV. Pintado et al. evaluated thallium SPECT in 33 patient with HIV infection and parenchymal brain lesions [106]. SPECT was positive in ten patients with lymphoma (five pathologically confirmed and five with a presumptive diagnosis) and was negative in 16 with non-tumoral lesions (ten with cerebral toxoplasmosis, three with progressive multifocal leukoencephalopathy, one with tuberculoma, one with pyogenic brain abscess and one with vasculitis). In seven patients the etiologic diagnosis of the brain lesions could not be established (SPECT was positive in two and negative in five). Simliarly Lorberboym et al. performed thallium SPECT in 18 patients with and found a sensitivity for lymphoma of 100% and a specificity of 90% [107].

The approach to the HIV-infected patient with parenchymal brain lesions is evolving. Because of the higher incidence of toxoplasmosis and the potential morbididty of brain biopsy, it has been common practice to treat patients with multiple CNS lesions empirically with anti-toxoplasmosis drugs and dexamethasone to reduce cerebral edema. Patients with no improvement after a week typically have undergone brain biopsy. In patients with a solitary, lesion on scan (CT of MRI), the diagnosis of lymphoma is more likely, and these patients have gone directly to brain biopsy [108]. With the advent of thallium SPECT scanning, this strategy may no longer be appropriate. Some practitioners now advocate initiating treatment for lymphoma without a brain biospy in patients with findings that strongly support the diagnosis: CD4+ lymphocyte count under 50 cell/μl, solitary brain lesion, positive imaging on thallium SPECT and a negative test for serum antitoxoplasmosis antibodies. In patients for whom significant doubt exists as to the diagnosis, a brain biopsy is indicated. It should be noted that in children infected with HIV, lymphoma is the most common cause of a focal CNS mass; although thallium SPECT may be useful in these patients, there is no data on its use in HIV-infected children.

The treatment of patients with primary CNS lymphoma is unsatisfactory regardless of the patient's HIV status. Conventional therapy with corticosteroids and cranial irradiation produces responses in most non-HIV-infected patients, but disease recurrence is common. Median survival is 12–18 months, and five-year survival is 3%–4% [109]. In HIV-infected patients, approximately 75% of patients demonstrate clinical improvement with radiation and corticosteroids, but median survival is only 3–5 months, and few patients survive more than a year [70–72]. Most patients die from opportunistic infections, reflecting the severe immunosuppression that accompanies primary CNS lymphoma in the setting of AIDS [72]. Neither the degree of neurologic

dysfunction nor the extent of disease seen on CT or MRI scan predict respons or survival [72]. Survival of untreated patients is approximately six weeks [72]

Because of the poor results of conventional therapy, the addition of chemo therapy has been investigated. DeAngelis et al. treated 31 non-HIV-infected patients with preradiation methotrexate and a full course of cranial irradiation followed by two cycles of high-dose cytarabine and achieved a median survival of 42.5 months, a significant improvement over the results of conventional therapy [110]. Similar results have not been obtained using combined therapy in patients with HIV infection. Forsythe et al. treated 10 patients with HIV infection and primary CNS lymphoma with chemotherapy (methotrexate thiotepa, and procarbazine) and cranial irradiation. Seven patients were evaluable for response: six patients responded, and all responses were deemed complete [111]. Median survival was 3.5 months. All three patients who survived more than six months experienced significant dementia, suggesting that HIV infection, which itself may cause dementia, may place patients at high risk for neurologic toxicity from therapy.

Standard therapy for HIV-associated primary CNS lymphoma remains corticosteroids and palliative cranial irradiation. Patients who receive at least 4000 cGy appear to have the highest likelihood of achieving a complete response and surviving more than six months [72,92]. The most important side effect of cranial irradiation is fatigue, which can be severe and may persist for a week or more after completion of therapy. Because of the overall poor survival, some patients may elect not to be treated and will be candidates for hospice care.

Conclusions

As the AIDS epidemic continues, HIV-related NHL remains a difficult problem. Advances in the treatment of opportunistic infections and antiretroviral therapy have not been matched by advances in the treatment of HIV-related NHL: although some patients achieve prolonged survival, most die of progressive lymphoma or complications of AIDS within a year of their lymphoma diagnosis. As shown in Figure 1, during the past 10 years, the immune status of the average patient treated on a prospective protocol for NHL has declined substantially. The lower CD4-positive lymphocyte counts now present at the time of diagnosis of NHL, reflecting a patient population whose care is often complicated by multiple medical problems and poor hematologic reserve. Definitive studies have shown that dose-intensive strategies do not benefit these patients; new, less toxic, but nonetheless effective regimens are needed.

The treatment of HIV infection is evolving rapidly: recent advances include the development of new assays to measure viral load and the availability of a new class of effective antiviral drugs, the HIV protease inhibitors. Combination antiretroviral therapy with a protease inhibitor and two reverse transcriptase inhibitors is substantially more effective than the therapie

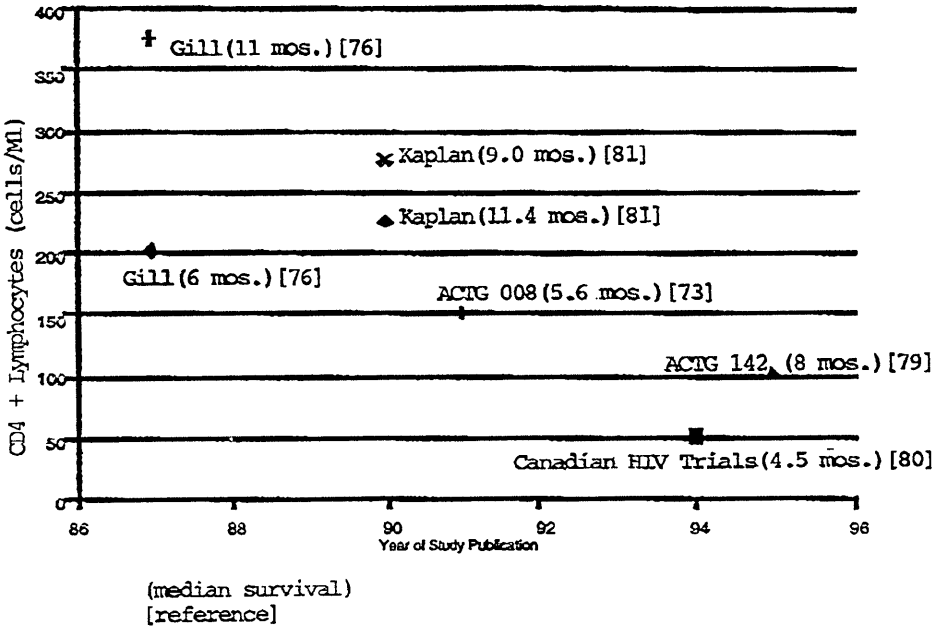

Figure 1. Prospective trials in AIDS-NHL.

previously available. The impact these strategies will have on the incidence of NHL remains uncertain. To the degree that new therapies result in patients with HIV infection living longer with chronic B-cell stimulation and immunosuppression, the incidence of NHL may rise. On the other hand, antiretroviral therapy that successfully decreases HIV viral load may also be effective in decreasing the risk of developing NHL.

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* For remainder of references, please see the Addendum to Chapter 10 at the end of this book.

11. Mechanisms of resistance against B-cell malignancies induced by vaccination against the immunoglobulin receptor: the case for T-cell immunity

Lars A. Sternas, Steve W. Weeks, and Larry W. Kwak

Immunoglobulin (Ig) molecules are composed of heavy and light chains that possess highly variable regions at their amino termini. These variable regions combine to form the antigen recognition site, which can itself be recognized as an antigen, termed the *idiotype*. B-cell malignancies are clonal proliferations of Ig-producing cells. The idiotypic determinants of the surface Ig can thus serve as a tumor-specific marker for the malignant clone. We and others continue to investigate strategies to induce specific immunity against the idiotypic determinants on the Ig molecule expressed and/or secreted by B-cell malignancies by active immunization. This chapter will summarize the progress in exploiting tumor-derived Ig protein as a tumor-specific antigen for therapeutic vaccine development and the priorities for future investigation.

Preclinical experimental models

The starting point for rational clinical trial design is an understanding of the effector mechanisms involved. Lynch and Eisen [1–3] were the first to demonstrate that syngeneic BALB/c mice immunized with purified myeloma Ig proteins derived from mineral-oil-induced plasmacytomas (MOPCs) developed an antibody response specific for the individual idiotype used for immunization. Furthermore, these animals were able to specifically reject a tumor challenge of the parental tumor but were not resistant to other MOPC tumors, thus demonstrating the specificity of this antitumor immunity for idiotype. In order to elicit these responses, the purified antigens required formulation with complete Freund's adjuvant. Subsequently, Jorgensen et al. [4] demonstrated that immunization with light chains alone from the MOPC-315 plasmacytoma was sufficient to confer protection against subsequent tumor challenge. Tumor resistance in this model system correlated with proliferation of spleen cells in vitro, without apparent antibody production.

Subsequently, the phenomenon of idiotype-specific protective antitumor immunity induced by Ig immunization was reproduced in a number of myeloma, leukemia, and lymphoma models [5–8]. Studies designed to elucidate cellular mechanisms of tumor resistance, however, have yielded mixed

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results, as exemplified by the BCL-1 and 38C13 lymphomas, in which tumor protection could be transferred by mixing serum from immune mice with the tumor cells prior to challenge of naive mice, suggesting a pivotal role for antibodies [6,8]. Nevertheless, protection against a subsequent tumor challenge could also be reversed by treating immunized mice with depleting mAb against T cells [8].

Additional insight into the role of T cells in the response against tumor idiotype has been obtained from experiments with cloned, idiotype-specific T cells. Immunization of mice with light chain derived from MOPC-315 elicited MHC class II-restricted CD4+ T-cell clones of both Th1 and Th2 subtype [9]. When either of these clones was injected subcutaneously, together with a challenge of syngeneic cells transfected with the cognate idiotype, each protected mice in an idiotype-specific and dose-dependent manner. To mimic more closely the in vivo situation, wherein naive lymphocytes are able to interact with target tumor cells immediately following activation and in the absence of preformed antibodies, mice transgenic for the T-cell receptor (TG-TCR) isolated from these T-cell clones were generated. These transgenic mice were resistant to challenge with the Ig-secreting tumor [10]. Furthermore, because the tumor was MHC class II negative, an indirect mechanism of tumor cell neutralization was postulated [11]. Finally, adoptive transfer of TG-TCR lymphocytes into SCID mice, devoid of B cells, produced resistance to challenge with MOPC-315, demonstrating that such idiotype-specific T cells were sufficient for tumor resistance.

The best evidence for CD8+ idiotype-specific T cells has been provided by the unexpected finding of cross-reactivity of murine influenza virus hemagglutinin-specific T-cell clones against an epitope nine amino acids long contained in the CDR-2 region of an Ig heavy chain variable region of a myeloma [12].

We recently used the 38C13 lymphoma model to test a series of selected recombinant cytokines for their ability to enhance the protective effect of immunization with tumor-derived Ig (Id) conjugated to keyhole limpet hemocyanin (KLH) [13]. It had been previously shown that conjugation to an immunogenic carrier protein, such as KLH, was required to elicit protection against a subsequent tumor challenge in this model system [7]. For these studies, a single dose of Id-KLH was injected subcutaneously, followed by free recombinant murine GM-CSF administered either locally as close as possible to the initial immunization site or systemically (intraperitoneally). GM-CSF was injected daily for four days, starting on the day of vaccination. Fourteen days later, immunized mice were challenged with 10,000 tumor cells (100 times the minimum lethal dose) and followed for survival. A representative experiment is summarized in Table 1 (Exp. 1). All four groups of mice receiving GM-CSF showed significantly prolonged survival as well as an increase in the number of long-term survivors, compared with mice immunized with the Id-KLH conjugate alone. Furthermore, GM-CSF administered locally (subcutaneously) was more effective than systemic (i.p.) administration, and a

Table 1. Testing of free cytokines enhance the protective effect of Id-KLH immunization

Exp.	Immunogen	Cytokine dose/schedule[a]	Tumor challenge dose	No. survivors/total no. mice	*p*-value[b] vs. Id-KLH alone
1	Id-KLH + GM-CSF	50,000 U d1–4, s.c.	10^4	2/10	0.001
		10,000 U, s.c.		4/10	0.001
		50,000 U, i.p.		2/10	0.003
		10,000 U, i.p.		2/10	0.002
	Id-KLH alone			0/10	—
	Control Igm-KLH			0/10	0.01
2	Id-KLH + IL2	50,000 U d1–5	10^3	4/10	NS[c]
		20,000 U		7/10	NS
		10,000 U		8/10	NS
		5,000 U		3/10	NS
	Id-KLH alone			4/10	—
	Control IgM-KLH			0/10	0.001
3	Id-KLH + IL-2	50,000 U d1–5	5×10^3	5/15	NS
		10,000 U		5/15	NS
	Id-KLH alone			5/15	—
	Control IgM-KLH			0/15	0.005
4	Id-KLH + IFNg	5,000 U d1	10^4	3/10	NS
		1,000 U d1–5		2/10	0.07
	Id-KLH alone			0/10	—
	Control IgM-KLH			0/10	0.022
5	Id-KLH + IL-1α	400 ng d1–8	5×10^3	2/10	0.05
		200 ng		6/10	NS
		100 ng		7/10	NS
	Id-KLH + 1β	400 ng		6/10	NS
		200 ng		4/10	NS
		100 ng		4/10	NS
	Id-KLH alone			4/10	—
	Control IgM-KLH			0/10	<0.001
6	Id-KLH (i.d.) + IL-12	0.1 mcg d1–4 i.d.	10^4	2/10	NS
	Id-KLH (i.d.) alone			0/10	—
	Id-KLH (i.p.) + IL-12	0.1 mcg d1–4 i.p.		0/10	NS
	Id-KLH (i.p.) alone			0/10	—
	Control IgM-KLH			0/10	<0.05

[a] The experimental design for each experiment was as follows: immunization with Id-KLH 50 mcg s.c. on day 1 (d1) plus cytokine administration i.p. (unless otherwise indicated) according to the schedule indicated, followed by tumor challenge i.p. two weeks later.

[b] Based on logrank analysis of survival curves, not only on absolute numbers of survivors.

[c] $p > 0.1$.

reproducible trend, approaching statistical significance, in favor of lower doses was observed. Representative experiments with other recombinant cytokines are also shown in Table 1 (Exps. 2–6). The addition of human IL-2, murine interferon-gamma, human IL-1 alpha or IL-1 beta, or murine IL-12 by various routes, doses, and schedules of administration described by others [14–18] failed to produce significantly augmented protection following tumor challenge, compared with a single subcutaneous immunization with Id-KLH alone. In addition, combinations of murine GM-CSF with IL-12, IL-2, interferon-gamma, or human TNF-alpha failed to demonstrate any additional protective effect compared with GM-CSF alone when given together with a single Id-KLH immunization (data not shown). The mechanism of the additive tumor protection produced by GM-CSF was investigated further. It was critically dependent upon effector CD4+ and CD8+ T cells, as demonstrated by the abrogation of protection in immunized mice after depleting either subset with monoclonal antibodies in vivo. This clear-cut requirement for T cells was not associated with any apparent increase in antibody response directed against idiotype, suggesting that the mechanism of augmented protective antitumor immunity by GM-CSF was dependent on the selective activation of the T-cell arm of the immune response.

Studies in human patients

The first study of Ig-KLH immunization in human patients with relapsed low-grade lymphomas demonstrated that specific antibody responses against autologous idiotype could be elicited in 7 of 9 patients [19]. Guided by our results above, a second larger clinical trial (42 patients), incorporating improvements in vaccine formulation and study design, was opened in 1994 [20]. Previously untreated patients with follicular lymphoma are first treated with modified ProMACE chemotherapy. After achieving a complete remission, these patients then receive a series of five monthly subcutaneous vaccinations with autologous Ig-KLH, together with locally administered free GM-CSF (patients are randomized to receive either 500 or 100 mcg/m^2). The specific aims of this study are 1) to demonstrate specific immunity against the individual idiotype expressed by each patient's tumor, and 2) to test whether idiotype vaccination can eradicate rearranged bcl-2 positive tumor cells remaining in the bone marrow after chemotherapy.

In contrast to the pilot study above, local reactions characterized by erythema and induration (at least 8 cm) have been consistently observed at the vaccination sites of all patients, starting with the second vaccination in the series. These local reactions were subsequently reproduced at distant sites by a challenge with unconjugated autologous Ig alone (without KLH), but not with GM-CSF alone, suggesting that a significant component of this response is directed against the autologous Ig. In 5 of 6 patients formally tested, this response was specific for the individual idiotype, as demonstrated by lack of a

reaction to a control, isotype-matched Ig. These early results provide convincing in vivo evidence that it is possible to induce a specific immune response against a self tumor antigen and that GM-CSF is a potent immunostimulant. Furthermore, 2 out of 3 patients analyzed thus far whose bone marrows contained residual rearranged bcl-2 positive tumor cells detectable by PCR postchemotherapy became PCR negative after vaccination.

Perhaps the best evidence that human Id-specific T cells can be elicited by vaccination has been provided by the initial experience in multiple myeloma. We immunized an HLA-matched sibling marrow transplant donor with Ig-KLH isolated from the plasma of the recipient and subsequently demonstrated proof of principle that an Id-specific T-cell response could be transferred to the myeloma recipient [21]. Following bone marrow transplantation, a donor-derived, CD4+ T-cell line was isolated from the recipient that was specific for the idiotype of the M-protein. The responsiveness of this line for Ig was blocked by antibodies specific for MHC class II, suggesting that T-cell recognition was against an idiotypic peptide in an MHC-restricted manner.

Conclusions and future directions

The phenomenon of protective antitumor immunity induced by immunization with B-cell tumor-derived Ig is well established in small animal models. An important role for T cells in the effector phase of the immune response is suggested by some of these experimental models. T cells appear to be particularly important in formulations that include free GM-CSF as an 'adjuvant.' Our goals for Id vaccine development are 1) to focus on formulations that are more effective in activating the cellular arm of the immune response in human patients, and 2) to further increase vaccine potency. Our working hypothesis is that achieving the eventual goal of clinical antitumor efficacy will depend on the ability of Id vaccines to elicit sustained, potent, cell-mediated responses.

The current trial of Id vaccination in previously untreated patients with follicular lymphomas (Id-KLH with locally administered free GM-CSF) has already provided conclusive proof for the cancer vaccine concept, i.e., simply that it is possible to induce a specific immune response against this self protein. The study of immune lymphocytes from patients on this trial is critical to our long-term goal of elucidating the structural basis of Id recognition by human T cells. Efforts are focused on establishing idiotype-specific T-cell lines and clones as unique reagents for mapping studies with defined tumor Ig variable region peptides. Such lymphoma and myeloma-derived Ig variable-region gene sequences are already being determined prospectively for individual patients. These proposed studies should elucidate the molecular basis of Id recognition by human T cells and may provide the necessary, definitive proof of their specificity for idiotype.

In parallel, we anticipate that the development of second-generation Id vaccines based on recombinant approaches will lead to improved vaccine formulations for clinical testing. Using techniques of PCR amplification and rapid sequencing of Ig variable-region genes, a focus of our laboratory research is the construction of Ig heavy and light chain variable-region gene fragments linked together at the DNA level by a short oligonucleotide linker (single chain Fv). Based on their potential to prime for an MHC class I or II restricted T-cell immune response to other model antigens, we have selected live delivery vehicles, such as Bacille Calmette–Guerin (BCG) and pox viruses, for expression of recombinant idiotype as live vaccines, as well as DNA vaccines, consisting of recombinant idiotype. It is noteworthy that neither idiotype-cytokine fusion proteins nor DNA vaccines expressing idiotype described to date have been demonstrated to induce any significant evidence of T-cell activation in animal models [22–24]. In addition, our studies with cellular lymphoma vaccines engineered to overexpress GM-CSF have shown potential for priming specific T-cell responses to tumor idiotype and have also shown potential for eradicating early established tumors [25]. These latter studies may provide the rationale for a future clinical strategy combining cellular and purified Id vaccines.

Several other considerations remain. First, conclusive demonstration of a human therapeutic T-cell response against idiotype will require the unequivocal demonstration that the autologous lymphoma or myeloma cell (or, in the case of myeloma, the clonally related precursor B cell) can serve as a target of such T cells. Secondly, the potential obstacle of T-cell tolerance, perhaps induced by circulating idiotypic antigen, particularly in the setting of advanced disease, needs to be more completely understood. Finally, streamlined methods for producing these patient-specific vaccines will be required for broad application.

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12. Novel biologic approaches to hematologic malignancies

Janice P. Dutcher and Peter H. Wiernik

Normal hematopoiesis and lymphopoiesis are dependent upon the interactions of a broad array of endogenous cytokines that have both positive and negative influences on proliferation and differentiation of these tissues. Many hematologic malignancies demonstrate aberrant growth or differentiation or both, yet appear to retain their ability in part to respond to normal regulatory molecules. Therefore, over the course of the past 20 years, attempts have been made to utilize regulatory cytokines to control hematologic malignancies. In certain instances, such as interferon therapy for hairy cell leukemia, this attempt has been quite successful, and in other situations we are still exploring the possibilities. Nevertheless, there appears to be a potential for growth control if not disease eradication through the utilization of biologic therapy in hematologic malignancies.

Biologic agents in the myeloid leukemias

Alpha interferon (IFNα)

Alpha interferon has been available for clinical study in a variety of forms for 20 years. It has a demonstrable antiproliferative effect on normal hematopoiesis and has a similar effect on some human hematologic neoplasms, such as myeloma and the blast form of chronic myelocytic leukemia (CML) [1]. In these diseases, the growth inhibition from IFNα is similar to that produced in normal cells, but the self-renewal capacity of malignant cells is markedly reduced [1]. The mechanism of this biologic effect on growth is not fully understood. Interferon-inducible enzymes can be detected that are believed to interact with cell-proliferation pathways [2]. More recently, effects of interferon on adhesion molecules in CML progenitor cells, such as restoring impaired β1 integrin receptor function, result in normal adhesion of myeloid cells to marrow stroma [3]. This outcome may be a mechanism of growth regulation mediated by interferon therapy of CML. Other interactions continue to be investigated.

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Chronic myeloid leukemia

CML is a disease for which conventional therapy has not made a major impact. However, interferon therapy appears to extend the life of patients, and in responders, to delay the time to development of the acute phase [4,5]. Talpaz et al. initially reported that therapy with either partially pure human leukocyte IFN or with recombinant IFNα produces complete hematologic remissions in greater than 70% of patients and cytogenetic remissions in greater than 40% of patients treated in the chronic phase of the disease [4,5]. These reports generated considerable excitement and led to trials evaluating dose and schedule, as well as the effect of cytogenetic response on outcome. In a randomized study, Alimena et al. demonstrated a dose–response effect, with those patients treated with IFNα2b 5×10^6 U/m^2 three times weekly having a significantly improved response rate compared to those treated at 2×10^6 U/m^2 three times weekly (47% vs. 24%, $p = 0.06$) [6]. In addition, 8 of 21 (38%) patients not responding to the lower dose and then treated with the higher dose achieved a complete hematologic response [6]. The Cancer and Acute Leukemia Group B (CALGB) experience in a phase II study demonstrated a 59% partial plus complete clinical response rate and a 40% cytogenetic complete plus partial response rate among 107 patients treated with IFNα2b at a dose of 5×10^6 IU/m^2 daily [7]. More than half of patients required some degree of dose reduction, but 58% received more than 50% of the target dose. The average dose administered was approximately 60% of that planned, but the time to cytogenetic response did not correlate with dose received [7]. Although an analysis of the survival data from this study suggested that the time to achieve cytogenetic response was in part the reason for reported improvements in outcome, the ability to respond to IFNα appeared to confer a better outcome.

Long-term responses have been documented by follow-up of the initial cohorts treated [8,9]. The M.D. Anderson experience has recently been updated, reviewing patients treated before 1988, with cytogenetic follow-up every three months in the first two years and then every 4–6 months. Durable cytogenetic responses were noted in approximately 30% of patients, ranging in length from 2 to 8 years [8,9]. The duration of cytogenetic response was longest in patients who achieved a complete cytogenetic response. Patients who stopped interferon and then developed recurrence of cells containing the Ph^1 chromosome had further responses to reinstitution of IFNα [9].

As a result of the evolving data from these and other phase II reports suggesting major activity of IFNα in CML, several international multicenter randomized studies have been initiated, and reports are now available (Table 1). The Italian Cooperative Study Group conducted a randomized study designed to detect a difference of 20% in median survival, comparing treatment in chronic phase with IFNα2a to treatment with conventional chemotherapy (hydroxyurea [HU] or busulfan [BU]) and has followed this group of over 300 patients for more than six years [10]. The time to progression was longer

Table 1. Randomized studies of IFNα in chronic phase of CML

Study design		Responses		Median TTP	Survival
		Heme	Cytogenetic		
					6yr
Italian [10]	IFNα	45%	8%CR; 11% PR	72mo	50%
(n = 322)	HU/BU	46%	0%CR; 1%PR	45mo	29%
			p = 0.005	p = 0.002	p = 0.002
					Median
German [11]	IFNα	92% CR + PR	7%CR; 2%PR	4.8yr	66mo
(n = 513)	HU	90% CR + PR	1.6% CR + PR	4.6yr	56mo
	BU	91% CR + PR	0.9% PR	3.15yr	48mo
				5-yr	5yr
Kouseisho [12]				cyto vs. no	
(n = 170)				cyto resp	
	IFNα	77% CR + PR	9% CR; 8% PR	70% vs. 22%	54%
	BU	97% CR + PR	3% CR; 3% PR	60% vs. 22%	32%
					p = 0.29
MRC [13]					
(n = 587)					
induction BU/HU					5yr
then	IFNα	87% CR + PR	6%CR; 5% PR	NA	52%
	BU/HU	—	0% CR; 2% PR	NA	34%

Abbreviations: HEME, hematologic; TTP, time to progression; IFN, interferon; HU, hydroxyurea; BU, busulfan; CR, complete response; PR, partial response.

among the patients treated with IFNα compared to conventional therapy (median 72 months vs. 45 months, $p < 0.001$), and survival was longer with IFNα (median 72 vs. 52 months, six-year survival 50% vs. 29%, $p = 0.002$ for both comparisons). Of interest, in this study only 8% had complete cytogenetic responses, but another 11% had greater than 67% reduction in Ph^1 chromosome [10]. The German CML Study Group has reported a randomized comparison of patients treated with BU, HU, or IFNα and describes a significant survival advantage for IFN-treated patients compared to BU-treated patients ($p = 0.008$), but no such advantage over HU-treated patients ($p = 0.44$) [11]. These authors hypothesize that the degree of myelosuppression that can be maintained, either by IFNα or by HU, impacts favorably on the long-term outcome. This study also reported that patients who continue IFNα survive longer than those who discontinue IFNα before blast crisis ($p = 0.007$). As observed in other reports, complete hematologic responders had a survival advantage over partial responders or nonresponders ($p = 0.02$) [11]. The Kouseisho Leukemia Study Group conducted a multicenter randomized trial in Japan comparing IFNα to BU in chronic-phase CML [12]. Using a landmark analysis to account for time in CML prior to study, they demonstrated that any degree of cytogenetic response conferred an improved survival advantage, and this outcome was true among patients treated with BU as well [12]. They concluded that IFNα induced a greater number of complete cytogenetic re-

sponses and, on that basis, showed enhanced survival and duration of chronic phase compared to BU [12]. The Medical Research Council of the United Kingdom has also completed a randomized trial of IFNαn1 in CML, and the initial report describes a survival advantage for IFNα-treated patients [13]. In all these studies, there is a consistent pattern of extension of chronic phase and prolongation of survival with the use of IFNα, and in several of the studies, this outcome correlated with achieving major cytogenetic responses. In all the above studies, time to achieving cytogenetic remission was prolonged, with a median of 12 months to achieve minor cytogenetic response and a median of 22 months to achieve complete response.

Accelerated CML

Of interest in the Kouseisho study, 11 patients were treated in accelerated phase (AP) or blastic phase (BP), and one of five in the IFNα group achieved a complete hematologic and another a partial hematologic remission [12]. Although early reports suggested that IFNα was ineffective in the advanced stage of CML, this report suggests that some patients may remain responsive. We have explored a regimen of IFNα and plicamycin in a phase II study in 13 patients with accelerated-phase CML, which has demonstrated substantial prolongation of postaccelerated-phase survival (median 23.5 months) in the entire group of patients treated [14]. The overall survival in accelerated phase is nearly as long as the time to development of accelerated phase. Clinical responses included stabilization of accelerating disease for prolonged periods of time (three patients) and no change but improved responsiveness to previously ineffective chemotherapy in three patients (median duration of stability after accelerated phase with this regimen, 21 months). There were also three partial hematologic responses and a complete hematologic response in one patient with a partial cytogenetic response, including loss of a secondary chromosomal abnormality (inv 3) (median duration 63+ months). Thus, the addition of plicamycin to interferon seems to enhance the response duration in advanced-stage patients. Recently, another report suggests that HU in conjunction with IFNα is synergistic in previously HU-resistant accelerated CML [15]. Of 10 patients treated with the combination, five achieved hematologic CR and one had a partial cytogenetic remission. All five patients are alive 1–3 years after treatment, continuing to receive IFNα and HU [15].

Other approaches in CML

Another approach that is generating increased interest is the combination of IFNα and low-dose cytarabine. Two groups have presented preliminary results demonstrating a higher complete hematologic response with that combination compared with IFNα alone [16,17]. Kantarjian et al. studied patients in late chronic phase/accelerated phase and reported a higher hematologic re-

sponse rate (55% vs. 28%, $p = 0.02$), a trend for better Ph^1 chromosome suppression (15% vs. 5%), and significantly longer survival (three-year survival rate 75% vs. 48%, $p < 0.01$) compared to historical controls treated with IFNα alone [17]. Guilhot et al. conducted a prospectively randomized study comparing IFNα with or without low-dose cytarabine and in preliminary results reported a significantly higher complete cytogenetic response rate for the combination (28% vs. 8%, $p = 0.01$) [16]. These data are currently being updated (Prof. F. Guilhot, personal communication).

Gamma IFN

The early success of IFNα in CML encouraged an evaluation of other interferons and of combinations of IFNα with other agents. Gamma interferon, also called immune interferon, has a different mechanism of action and utilizes a different receptor. In vitro studies have shown an antiproliferative effect in CML [18]. Clinical studies demonstrated hematologic responses in approximately 20% of patients [19], but studies of combinations of gamma and alpha interferons have not shown synergy [20,21].

All-trans retinoic acid (ATRA)

ATRA is a major new therapeutic option in the treatment of acute promyelocytic leukemia [22,23]. We have also demonstrated activity for this agent in CML, first in a single patient with a morphologically promyelocytic blast crisis of Ph^1-positive CML [24]. That patient had failed interferon and developed blast crisis, manifested as fever, increasing splenomegaly, circulating blasts, and thrombocytopenia. His marrow was also blastic. Because of his morphology, despite not having the t(15;17) translocation, he was treated with oral ATRA and remarkably had a major clinical response with resolution of fever and splenomegaly and normalizaton of his peripheral blood counts. He remained in this chronic phase for several months [24]. Gallagher et al. studied his blast cells and demonstrated maturation in response to retinoids in vitro [25]. In ongoing studies of patients with CML, these authors have subsequently demonstrated that granulocyte–macrophage colony forming units (CFU-GM) from additional patients with advanced-stage CML (blastic or accelerated phase) were inhibited by ATRA approximately 1000-fold more effectively than CFU-GM from chronic-phase CML patients [25]. This differential sensitivity was also noted with *cis* retinoic acid in vitro. In these studies, in contrast to findings in human AML cells [26], there was very little to no interaction in inhibition between ATRA and IFNα. Research into the mechanism of the action of retinoids in advanced CML will be correlated with clinical results of an ongoing Eastern Cooperative Oncology Group clinical trial of ATRA in accelerated/blastic CML (study E1993).

Donor leukocyte infusions

Allogeneic bone marrow transplantation (BMT) has yielded long-term survival and even cure in many younger patients with CML. However, relapse of this highly resistant disease remains a problem. Allogeneic BMT may in part be effective due to the development of a graft-versus-leukemia (GVL) effect induced by the donor immune cells. Several investigators have recently reported a salutary effect upon relapse following allogeneic BMT by altering the immunosuppression. An early report of abruptly stopping the immunosuppressive medication demonstrated rapid reversal of relapse with both complete hematologic and cytogenetic responses in two patients [27]. Both patients developed signs of graft-versus-host disease (GVHD) as well, but this resolved and they remained in complete remission at the time of the report. Others have reported the beneficial effect of donor leukocyte infusions with or without IFNα to induce a GVL effect in relapsed transplant patients and have documented the achievement of molecular remission following these infusions [28–32]. The degree of GVHD does not necessarily correlate with the success of GVL. This immunomodulation appears to be a successful alternative to a repeat BMT. This approach has recently been reported to have some effect in AML but apparently to a lesser degree [33].

Juvenile chronic myelogenous leukemia

Juvenile chronic myelogenous leukemia (JCML) is a rare leukemia of infants and young children, with a rapidly progressive course. There is no effective therapy other than allogeneic BMT. In in vitro studies, IFNα has been shown to suppress growth of JCML progenitors [34], but it has not been successful clinically. Retinoids also inhibit in vitro growth of progenitor cells from JCML patients, and in one report there was 60%–100% inhibition of spontaneous proliferation of GM-CFU [35]. Therefore, a pilot study of isotretinoid in JCML was initiated. Of 10 evaluable patients, two had complete responses, three had partial responses, and one had a minimal response. Of the six responders, five are stable, one is in CR without BMT, two are in CR with BMT, and two partial responders are stable on continued isotretinoin therapy [35]. This remarkable outcome in this uniformly fatal disease has led to subsequent studies, which are ongoing.

Future directions in CML

Hematopoietic colonies from patients with CML cultured in vitro retain some requirements for growth factor stimulation. However, precursors from patients who have become resistant to IFNα appear to have more autonomous growth and do not require the array of growth factors that IFNα-sensitive CML precursors do. However, the resistant colonies do require accessory

cells, and Estrov et al. have demonstrated that these accessory cells produce high levels of IL-1β, a cytokine important in hematopoiesis [36]. Subsequent studies demonstrated an inhibitory effect of soluble IL-1 receptor (sIL-1R) and IL-1 receptor antagonist (IL-1RA) on the growth of IFNα-resistant CML cells, suggesting that IL-1 is a mediator of the otherwise autonomous growth [36]. More recently, this group has correlated IL-1β levels with the stage of CML, with much higher levels present in patients in accelerated/blastic phase than in patients in chronic phase [37]. Patients with accelerated/blastic disease also had lower levels of naturally occuring IL-1RA. Thus, there seems to be dysregulation of both IL-1β and IL-1RA, and this group is initiating early studies to block IL-1R in an attempt to regulate CML growth in vivo. They have observed similar effects in the blasts of acute myeloid leukemia patients, also, and this approach may lead to clinical trials in both diseases [38]. Interestingly, IFNα and IFNγ both reduce IL-1 synthesis [39,40], and this may in part account for the suppressive effect of IFNs.

Acute myeloid leukemia (AML)

Interleukin-2

Interleukin-2 (IL-2), a cytokine initially known as T-cell growth factor, is secreted from antigen-activated T cells that then induce activation of additional antigen-sensitized lymphocytes in a directed immune response [41,42]. IL-2 also induces an antigen-nonspecific activation that includes natural killer cells, lymphokine-activated killer (LAK) cells and monocytes, with release of numerous other cytokines that all participate in the immune response [43]. IL-2-activated T lymphocytes and LAK cells are capable of killing tumor cells and are directly cytotoxic against human hematologic malignancies, including against fresh human leukemia cells [44–48]. In addition, patients with active leukemia are able to generate IL-2-activated killer cells that are capable of a cytotoxic response against autologous and allogeneic human leukemic cells [49–51]. Additionally, patients with leukemia treated with IL-2 have demonstrated in vivo induction of killer cell activity [51].

Early clinical trials have demonstrated antileukemic responses in patients receiving IL-2 therapy [52–54] (Table 2). Patients with less advanced leukemia appeared to fare better [52] and in a recent study, 8 of 14 patients in partial remission (<25% blasts) who were then treated with high-dose IL-2 proceeded to complete remissions, some durable (Table 2a) [54]. Others have utilized IL-2 therapy for high-risk patients already in complete remission as a means of treating minimal residual disease [55–57] (Table 2b). Two such studies suggest a beneficial result in prolonging the time to relapse in patients in second remission, with a greater number of patients having second remissions longer than their own first remissions, compared to the number expected from historical controls (43% vs. 20%) [56,57]. In our study, two patients with

Table 2. Phase II studies of IL-2 in AML

A. Studies in active leukemia

Study/schedule	# pts	%blasts	Response	Duration (mo)
Foa [52] 100–1000 ug/m² × 24 hrs × 5 d × 2 wks, 4 cycles	5 7	8%–15% >20%	3 CR 0	4–30+
Maraninchi [53] 8 × 10^6/m² every 8 hr × 5 d every 2 wks × 3 cycles	10	13%–85%	2 CR, 1 PR	3, 3, 4
Meloni [54] 8–18 MU/m²/d IVCI × 5 d × 4 cycles then 4–8 MU/m² × 5 d each mo × 1 yr, then every 2 mo	14	>5%–<30%	8 CR, 1 PR 1 transient	CR duration: 2, 4, 5, 14+, 30+, 32+, 33+, 68+

Abbreviations: pts, patients; IL-2, interleukin-2; ug, microgram; MU, million units; IVCI, intravenous continuous infusion; CR, complete remission; PR, partial remission.

B. Studies of IL-2 maintenance in AML

Study/schedule	# pts	CR Status	Outcome	Duration
Wiernik [56] PEG IL-2 1 MU/m² weekly IV over 15 min	7	2nd CR	3/7 2nd CR >1st CR 1st CR 4–22 mo, 2nd CR 4–72+ mo	1st/2nd CR 1. 4 mo /10 mo 2. 7 mo/75+ mo 3. 22 mo/68+ mo
Bergmann [57] 9 MU/m² IV inf over 1 hr, d1–5, 8–12 every 6 wks	21	2nd CR	5/21 2nd CR >1st CR	med DFS 11 mo (r 4–49+ mo)
Macdonald [58] after 3 cycles CT, 3 MU/ m²/24 hr × 120 hr, every 3 wks	9	1st CR	6/9 relapsed	med TTP 39 wks from CR (r 30–120 wk); 27 wks from IL-2 (r 2–51 wk)

Abbreviations: pts, patients; PEG IL-2, polyethylene glycolated interleukin-2; MU, million units; IV inf, intravenous infusion; DFS, disease-free survival; CR, complete remission; TTP, time to progression.

first remissions of 7 and 22 months continue in second remissions (maintained for one year with IL-2) greater than 6 and 5.5 years, respectively [56].

One caveat is that three recent reports have raised concern regarding AML FAB type M5. Three patients appeared to progress rapidly, and in FAB M5, IL-2 appears to induce overexpression of the alpha chain of the IL-2 receptor, and this may mediate a proliferative response [58–60]. However, small numbers of high-risk patients were treated, so it is difficult to draw conclusions at

this time. One can only recommend careful monitoring of all clinical parameters prior to beginning IL-2 and suggest looking for receptor upregulation in treated patients. An ongoing Eastern Cooperative Oncology Group trial will evaluate these parameters (study E5996).

Bone marrow transplantation — graft vs. leukemia

Recent laboratory data suggest that the mechanisms and specific cell types that induce GVHD and those that mediate GVL effect are not identical, and in fact can be sorted out [61–66]. Some in vitro studies suggest that IL-2 may be instrumental in inducing the GVL effect, and studies by Sykes et al. suggest that IL-2 may have an inhibitory effect on GVHD by preferentially interacting with specific subgroups of T cells [62,65].

The major complication of auto-BMT and T-cell-depleted allo-BMT is relapse, presumably because of the diminished incidence of GVHD and GVL. Murine leukemia models have utilized IL-2-activated bone marrow for transplantation followed by additional systemic administration of IL-2 [67,68], and T-cell depletion followed by IL-2 [69] and suggest that IL-2 does confer benefit in preserving GVL.

Despite the aggressive immunosuppressive regimens utilized for BMT, patients are able to generate activated immune cells posttransplant [70–73]. This, in fact, occurs even without IL-2 therapy, giving credence to the hypothesis that cytokine activation is a result of BMT in general [74,75]. Therefore, pilot clinical trials have begun and have shown that IL-2 with or without LAK cell infusions can be administered safely [76–79]. Early reports suggest that posttransplant IL-2 may prolong the duration of remission of patients undergoing autologous BMT, particularly in second or greater remission or in the setting of T-cell-depleted allogeneic BMT [77–79]. Further studies are ongoing.

Other immunomodulators in AML

Additional new biologic agents that have proven to be useful in the treatment of acute myeloid leukemia include the hematopoietic growth factors discussed in chapter 8 and all-*trans* retinoic acid — specifically for acute promyelocytic leukemia — discussed in chapters 4 and 5.

New agents being evaluated to stimulate the immunologic response against minimal residual disease include linomide, a quinolone derivative that enhances T-cell, NK-cell, and monocyte/macrophage activity [80,81]. Early data have shown immune augmentation in patients with acute leukemia and CML, with quantitative increases in NK cells and monocytes [82,83]. Pilot and randomized clinical trials of linomide following bone marrow transplantation in AML and CML are under way.

Bestatin (Ubenimex) is a small-molecular-weight dipeptide that binds to aminopeptidases located on the surface of immune cells and modulates cellu-

lar immunity [84]. Two randomized studies comparing bestatin administered with postremission maintenance chemotherapy versus chemotherapy alone have been reported [85,86]. In the earlier study, the treated group had a significantly longer survival [85], whereas in the second study there is a significant prolongation of remission duration [86]. A more recent report in which patients received nearly one year of consolidation/maintenance chemotherapy postremission and were then randomized to bestatin versus nothing showed no added benefit from bestatin following extensive chemotherapy in extending the duration of remission [87]. The difference in the timing of adding this agent may explain the differences between this study and the previous ones, but the overall role of this agent in postremission therapy is not yet defined. The early results remain intriguing.

Monoclonal antibodies will be discussed in detail in the section on lymphoma, but the techniques utilized to develop monoclonal antibodies directed toward B-cell-specific antigens are being adapted to other hematologic malignancies. The Memorial Sloan–Kettering Group has developed an antibody directed against CD33, an antigen present on most myeloid leukemic cells but also on monocytes and hematopoietic progenitors. The antibody, M195, does not, however, react with the pluripotent stem cell. They have completed a phase I study that demonstrated targeting [88] and in a subsequent phase II trial with ^{131}Iodine-labeled M195, then showed an antileukemic effect [89]. Pancytopenia was often profound and dose dependent. New studies are ongoing with humanized M195 antibody in patients with CML in relapse as preparation for BMT [90], and as treatment of minimal residual disease in acute promyelocytic leukemia [91].

Myelodysplasia

Myelodysplasia (MDS), discussed in detail in chapter 14, is a grouping of disorders believed to result from dysregulation of hematopoiesis with uncoupling of proliferation and differentiation [92–95]. MDS is characterized by profound cytopenias and clonality, usually with cytogenetic abnormalities, and eventual progression to acute leukemia [93,95]. MDS progenitors demonstrate defective proliferation, suggested to be due to defective responsiveness to hematopoietic growth factors; in some patients, elevated levels of growth factors are noted [96,97]. In vitro studies have suggested that some MDS progenitors retain the ability to respond to normal regulatory and differentiating signals [95].

To test this hypothesis in vivo, a series of clinical investigations of hematopoietic growth factors have been completed and have been reviewed in detail by Ganser and Holzer [98]. Their review outlines these potential benefits being evaluated: 1) stimulation of residual normal precursors, 2) differentiation of cells retaining ability to respond, 3) enhancement of chemosensitivity, or 4) supportive care following intensive cytotoxic chemotherapy [98].

Since the major complications of MDS are infections due to prolonged neutropenia, as well as complications of transfusions or bleeding due to symptomatic anemia and thrombocytopenia, the initial goal of growth factor treatment is clinical benefit, with a secondary goal of altering the disease process. From a review of the numerous studies available, it appears that the latter is not affected. However, in the majority (7%–80% in phase II reports) of patients receiving the lineage-specific growth factors, granulocyte (GCSF) or granulocyte–macrophage (GM-CSF) colony stimulating factors, there is improvement in absolute neutrophil count, sometimes to near normal, with a very small incidence of proliferation of blast cells induced by these agents [98]. At least one randomized study comparing GM-CSF to supportive care showed a sustained improvement in neutrophil count, with a substantial decrease in infection rate when GM-CSF was administered [99]. No other cell lineages improved. Studies of the combination of GM-CSF and low-dose cytarbine have not shown improvement over cytarabine alone [98].

Erythropoietin has been employed to reduce the transfusion requirement in MDS by giving pharmacologic doses to overcome what appears to be resistance to the endogenous hormone. A small proportion of patients in single-arm studies appear to have a response in terms of increase in reticulocytes (by 23%) and hemoglobin (by 17%), and in approximately 23% there is a reduction in red cell transfusion requirement [98]. This approach is lineage specific, with no benefit incurred to other cell lines. One small randomized study of erythropoietin versus control was negative, however [100], and perhaps larger randomized studies will help to identify the less than 25% of MDS patients who may actually benefit from erythropoietin.

Thrmbocytopenia remains a major clinical problem in MDS patients, and it was hoped that some of the newer, less lineage-specific growth factors would enhance platelet counts [101]. There was also concern, however, that they might induce leukemic proliferation. Small phase II studies of interleukin-3, a multilineage growth factor, were initially encouraging, with MDS patients showing multilineage improvement (>50% improvement in neutrophils, 33% in platelets, 25% in reticulocytes) and approximately 10% showing an increase in blasts [102–104]. Patients remaining on IL-3 had stable counts and the expected natural history of their disease. However, this agent may not be clinically available as new lineage-specific platelet growth factors enter clinical trials.

Currently, three such agents are undergoing preclinical and clinical investigation: interleukin-11 [105–107], megakaryocyte growth and development factor (MGDF) [108–110], and thrombopoietin [111–113]. All show promise in stimulating platelet recovery. Investigations to determine the degree to which such agents may affect the proliferation of other cell lineages as well as leukemic and myelodysplastic precursors are ongoing [112–114]. Clinical trials have begun and are of great interest.

Evaluation of differentiating agents in MDS has been somewhat disappointing, particularly in view of the success of these agents in more advanced

leukemia. Two studies of all-*trans* retinoic acid have demonstrated minor responses accompanied by substantial toxicity, and this agent was not recommended for further evaluation in MDS [115,116]. In contrast, earlier studies of 13-*cis* retinoic acid have reported hematologic responses including complete remissions, but with little impact on overall survival [117–119]. A randomized study of 13–*cis* retinoic acid versus supportive care was less encouraging [120].

Thus, the biologic therapies that appear most useful currently in MDS are the hematopoietic growth factors, either alone or as supportive care following intensive chemotherapy. Recent reports describe higher response rates for intensive chemotherapy in MDS, which has stimulated renewed interest in this approach [121,122]. In addition, GM-CSF has been shown to have a salutory effect on the outcome of elderly patients treated for AML [123]. Thus, utilization of growth factors as supportive care in MDS patients treated with chemotherapy should be useful. Additional exploration of regulatory cytokines, as a better understanding of the steps of growth control evolves, may eventually hold promise in MDS.

Biologic agents in B-cell malignancies

Myeloma

Interferon alpha has been investigated in the treatment of myeloma for the past 10 years. In initial studies of advanced patients, activity was noted, and in previously untreated myeloma patients, Quesada reported partial or better responses in 50% of a small number of patients treated, with a median response duration of 14 months [124]. However, with this relatively high dose of IFNα, the toxicity was considerable, and most patients were unable to sustain prolonged therapy. Subsequent studies of IFNα during induction therapy have added this agent at lower doses to standard chemotherapy [125,126]. In an Italian study, patients were randomized to melphalan and prednisone (MP), with or without IFNα2a at a dose of 5 MU three times per week for the first two weeks of induction chemotherapy [125]. In this report, the response rate was higher among the patients receiving IFNα (95% vs. 68%, $p < 0.05$), and the response duration was prolonged ($p < 0.025$). The median survival was 80 weeks in the chemotherapy-alone group and 90 weeks in those receiving IFNα in addition ($p < 0.025$) [125]. A subsequent study by the Nordic Myeloma Study Group also randomized patients to induction MP with or without IFNα, 5 MU three times weekly [126]. In this study, the response rate was identical in both arms (45% vs. 44%). However, response duration and plateau-phase duration were longer for patients receiving MP plus IFNα than for those receiving MP alone ($p < 0.05$), with a difference in median duration of 5–6 months. There was no difference in overall survival (29 months for MP alone

vs. 32 months for MP + IFNα) [126]. In both studies, patients underwent dose reductions due to interferon toxicity. Other studies that included INFα during induction have shown less definitive effect [127].

Based on in vitro studies of the effects of IFNα on myeloma cell growth, in which both colony formation and labeling index are decreased [128,129], there has been interest in utilizing IFNα as maintenance therapy following induction therapy in order to extend the plateau phase of the disease and perhaps have an impact on response duration and survival. Several randomized studies have been reported, and long-term followup is ongoing (Table 3) [130–135]. The initial report was the Italian study, which utilized IFNα at 3 MU/m² three times weekly as maintenance therapy in patients who had had some degree of response to myeloma with chemotherapy [130]. The duration of response from time of randomization was prolonged in the group of patients who received IFNα compared to those who did not (26 months vs. 14 months, $p = 0.0002$) [130]. Patients who had a substantial objective response to chemotherapy appeared to also have prolonged survival with the addition of maintenance IFNα compared to the untreated group ($p = 0.0352$). Three additional studies, one by the Nordic Cooperative Group, one by the Canadian Cooperative

Table 3. Randomized studies of maintenance IFNα in myeloma

		IFNα vs. OBS		
Study [ref]	Induction	Dose IFNα	TTP	Survival
Italian ($n = 101$) [130]	MP or VMCP or VBAP or VMCP/VBAP	3 MU/m² tiw	26 mo vs. 14 mo $p = 0.0002$	52 mo vs. 39 mo $p = 0.0526$ responders to induct chemo $p = 0.0352$
Nordic ($n = 125$) [131]	MP	5 MU tiw	plateau 13.9 mo vs. 5.7 mo $p < 0.0001$	NA
NCI Canada ($n = 176$) [132]	MP	2 MU/m² tiw	17 mo vs. 12 mo $p = 0.003$	43 mo vs. 35 mo, $p = 0.16$ adjusted to PS, 44 mo vs. 33 mmo, $p = 0.049$
Austrian ($n = 95$) [133]	VMCP	2 MU tiw	17.6 mo vs. 8 mo; $p < 0.02$	NS
SWOG ($n = 193$) [134]	VMCP/VBAP VAD; VMCP (P)/ VBAP (P)	3 MU tiw	12 mo vs. 11 mo; NS	NS
German ($n = 117$) [135]	MivP; VBAM Dex	5 MU tiw	13 mo vs. 13 mo; NS	NS

Abbreviations: IFNα, interferon alpha; TTP, time to progression; M, melphalan; P, prednisone; V, vinblastine; C, cyclphosphamide; B, carmustine (BCNU); A, Adriamycin; D/Dex, dexamethasone; MU, million units; tiw, three times weekly; PS, performance status.

Group, and one by the Austrian Cooperative Group, have also demonstrated significant prolongation of the plateau phase and time to progression with interferon maintenance but no difference in survival between the two groups (Table 3) [131–133]. The Canadian study, when adjusted for clinical prognostic factors, showed a trend toward a survival advantage [132]. Two other studies have demonstrated no significant impact of maintenance IFNα upon response duration, with very little difference between the two arms [134,135]. The SWOG study analysis suggested that high doses of corticosteroids were the most beneficial component of the therapy [134]. The explanation for these differences in outcome is not clear. At the present time, a meta-analysis of the raw data from all these studies is ongoing in order to determine whether clinical or treatment factors may have weighted the results of either the positive or negative studies.

Erythropoietin

Anemia is a major complication of myeloma, and multiple red cell transfusions are a component of supportive care of these patients. Two randomized studies have recently reported benefit from erythropoietin (EPO) in some myeloma patients with reduction of transfusion requirements [136,137]. In the study performed at the Mayo Clinic, in the initial double-blind phase, of the 10 patients treated with EPO, six had a complete response and one a partial response [136]. Subsequently, the 10 control patients were treated with EPO; three had a complete response and one a partial response. In a study by the European Study Group, patients were randomized to receive EPO on a fixed dose of 10,000 U/day, on a dose-escalation schedule beginning at 2000 U/day, or no EPO [137]. In both the fixed-dose and the escalation-schedule groups, there was a reduction in transfusion requirement and rise in hemoglobin in 64% and 70% of patients, respectively [137]. The recommended dose from this study is 5000 U/day. A daily dose schedule has not yet been compared to the three times weekly schedule used in the Mayo Clinic study.

Other agents in myeloma

Interleukin-2 has been studied in multiple myeloma, although not to the extent that IFNα has been. A recent report from the German Myeloma Treatment Group of low-dose IL-2 in advanced disease describes both response and stabilization among a small number of patients [138]. Six of 17 patients demonstrated response, and four others showed stable disease. Of these 10, four have continued on IL-2 for more than a year. The median survival of nonresponders was 12 months but was not reached for responders at 36 months at the time of the report [138]. Further evaluation of this agent in myeloma seems warranted.

We documented the clinical and laboratory response of two patients with myeloma to cyclosporine A (CSA), one in conjunction with chemotherapy and

one with CSA alone [139]. The first patient was refractory to anthracycline-containing chemotherapy but was given an additional cycle of chemotherapy with CSA. He then responded with a reduction in his paraprotein and resolution of his transfusion requirement that lasted two months. His myeloma cells did not express P-glycoprotein, so its inhibition is not the explanation for his improvement. The second patient was previously untreated but refused chemotherapy or corticosteroids and was treated with CSA alone. This patient demonstrated a marked improvement in her transfusion requirement, platelet count, and bone pain and a significant reduction in serum levels of beta-2 microglobulin, C-reactive protein, and interleukin-6 [139]. Our hypothesis is that CSA, by inhibiting T cells, caused the observed reduction in IL-6 levels, since IL-6 acts as an autocrine/paracrine growth factor in myeloma [140–142].

Thus, myeloma seems a disease sensitive to regulation by cytokines and growth-regulatory agents. Further evaluation of these agents alone or in conjunction with chemotherapy may provide a means of prolonging response and perhaps survival. The addition of these agents to the more aggressive approaches described in chapter 7 may also offer benefit.

Non-Hodgkin's lymphoma

As mentioned previously, IFNα has a major antiproliferative effect on hematopoietic progenitors [1,2]. IFNα is also antiproliferative to lymphoid progenitors and enhances differentiation of B cells and T cells [143,144]. IFNα modulates major histocompatibility antigen expression on lymphoid cells, which may affect immune recognition of tumor [144]. Mechanisms of IFNα activity in lymphoma may include both immune-mediated cytotoxicity and direct antitumor, antiproliferative effects [144].

IFNα has clinical antitumor activity in human B- and T-cell disorders, originally described in hairy cell leukemia, in which there were complete hematologic responses with correction of cytopenias, as well as substantial reductions in splenomegaly and marrow hairy cells [145,146]. IFNα also has major activity in T-cell leukemias, including cutaneous T-cell lymphoma, discussed in detail in chapter 9. In addition, the combination of IFNα with antiviral agents has produced complete responses in HTLV-1-associated T-cell lymphoma with extended survival [147,148]. This treatment obviously deserves further study.

Early studies in favorable-histology B-cell lymphomas have also demonstrated antitumor activity [149,150]. Response rates of 10%–45% have been reported, including complete responses, but at the cost of significant toxicity due to the doses required [149,150]. However, the activity observed has led to a second generation of studies in which IFNα, at lower doses and intermittent schedules, is combined with standard antilymphoma chemotherapy (Table 4). In a study by the Eastern Cooperative Oncology Group (ECOG), in patients

Table 4. Randomized studies of chemotherapy +/− IFNα in non-Hodgkin's lymphoma

Study [ref]	Histology	Chemo	IFNα	TTP	Survival
EORTC $n = 331$ [155]	WF B, C	COP + RT	3 MU tiw after chemo	135 wk vs. 86 wk $p = 0.02$	NA
ECOG $n = 291$ [151,152]	WF A, B, C, D	COPA	6 MU/M^2 d 22–26 chemo each cycle	2.5 yr vs. 1.6 yr $p < 0.001$	at 3 yr fu, $p = 0.014$ at 5.25 yr, NS
GELA $n = 242$ [153]	WF B, C	CHVP × 6 mo then every 2 mo × 12	5 MU tiw for 18 mo with chemo	34 mo vs. 19 mo $p < 0.001$	3-yr survival 86% vs. 69%
German low-grade NHL group $n = 498$ [154]	WF B, C	COP or PmM	5 MU tiw after chemo	37 mo vs. 20 mo $p = 0.0033$	4 yr DFS 49% vs. 27%

Abbreviations: Chemo, chemotherapy; IFNα, interferon alpha; TTP, time to progression; EORTC, European Organization for Research in Treatment of Cancer; ECOG, Eastern Cooperative Oncology Group; GELA, Groupe d'Etude des Lymphomes de l'Adulte; NHL, non-Hodgkin's lymphoma; WF, Working Formulation; C, cyclophosphamide; V, teniposide; O, oncovin (vincristine); P, prednisone; A, Adriamycin (doxorubicin); H, hydroxydaunorubicin (doxorubicin); Pm, prednimustine; M, mitoxantrone; MU, million units; tiw, three times weekly; DFS, disease-free survival; NA, not available; NS, not significant.

with intermediate-grade non-Hodgkin's lymphoma (NHL), patients were randomized to receive cyclophosphamide, vincristine, prednisone, and doxorubicin (Adriamycin) (COPA) in a 28-day cycle, with or without IFNα given on days 22–26 [151,152]. Response rates were identical, but despite a reduced dose intensity in the patients receiving IFNα, the response duration among the patients receiving COPA plus IFNα (I-COPA) was significantly prolonged compared with those receiving COPA alone [151,152]. A recent update of this study confirms the significantly prolonged time to treatment failure ($p = 0.0013$ at 5.25 years of follow-up) and response duration (CR median 1.7 years for COPA vs. not reached for I-COPA) [152]. There is also longer survival among the I-COPA group, although this was not statistically significant. Several additional randomized studies have been performed, including a report from the French Groupe d'Etude des Lymphomes de l'Adulte (GELA) [153]. This study treated patients with low-grade follicular lymphoma with cyclophosphamide, doxorubicin, teniposide, and prednisone (CHVP), monthly for six months, and then every two months for a year [153]. Patients were randomized to receive IFNα at a dosage of 5 MU three times weekly for 18 months during the chemotherapy. In this study, patients receiving CHVP + IFNα had a statistically significant increased response rate (85% vs. 69%, $p = 0.006$), a longer median event-free survival (34 months vs. 19 months, $p < 0.001$) and a

higher rate of survival at three years (86% vs. 69%, $p = 0.02$). Eleven percent of patients stopped IFNα for toxicity [153]. The German Low Grade NHL Study Group has performed a randomized study in which patients were treated first with cytoreductive chemotherapy with cyclophosphamide, vincristine, and prednisone (COP) or prednimustine and mitoxantrone (PmM). Patients were then randomized to IFNα maintenance (5 MU three times weekly) or to observation [154]. Interferon was continued until relapse. Response rates to the two induction therapies were identical (85% vs. 86%) but with more complete responses to the PmM regimen (33% vs. 22%, $p < 0.05$) [154]. In the IFNα group, the estimated median disease-free survival (DFS) is 37 months versus 22 months for control utilizing the Logrank test ($p = 0.0033$), and the projected DFS at four years is 49% versus 27%. Because of these early results, the second randomization has been discontinued in favor of IFNα maintenance [154]. Further maturation of the results will be followed. The EORTC Lymphoma Cooperative Group has also conducted a randomized study in stage III/IV low-grade NHL in which patients were first treated to best response with COP followed by radiotherapy, and then randomized to 12 months of IFNα (3 MU three times per week) or observation [155]. The initial report of these authors at two years median follow-up, six and a half years after initiation of the study, showed median progression-free survival of 135 weeks for the IFNα group versus 86 weeks for the control group ($p = 0.02$) [155]. A recent presentation shows no survival advantage, now four years later.

Thus, IFNα appears to consistently prolong remission duration and time to treatment failure at a moderate and tolerable dose in conjunction with effective chemotherapy for low-grade lymphoma. This approach has been adopted enthusiastically in Europe, although less so in the United States. However, as discussed below, immunomodulation is currently under investigation as post-BMT therapy of minimal residual disease and to induce GVL.

Interleukin-2 in NHL

IL-2 has been utilized as a single agent in phase II studies of patients with relapsed NHL. Results have shown occasional responses, including complete remissions, but no major activity in these heavily treated patients [156–160]. Additional studies with combination regimens or with chemotherapy have not been reported.

Interest has been generated, however, in utilizing IL-2 or IFNα as postautologous BMT therapy to treat minimal residual disease and to induce a graft-versus-lymphoma effect [78,161–164]. Studies to date are essentially feasibility studies, showing that patients tolerate these agents following autologous BMT [161–164]. However, in two therapeutic studies of IL-2 reported, there appears to be a salutary effect on duration of response, although populations are quite heterogeneous [78,163]. Randomized studies are pro-

posed to further investigate these potential options for extending duration of response following auto-BMT.

Interleukin-4

IL-4, initially described as B-cell growth factor and later called B-cell stimulatory factor [165,166], enhances B-cell proliferation in response to antigens or to anti-IgM [165]. IL-4 induces differentiation of normal B cells, plays a major role in inducing immunoglobulin shifts with increased production of IgE, and promotes proliferation after activation [167–171].

The effect of IL-4 on B-cell malignancies in vitro seems to be mediated by multiple pathways utilized to produce or inhibit the effects of differentiation and proliferation [172,173]. Taylor et al. have shown growth inhibition by IL-4 during culture of cells from patients with low-grade lymphoma and myeloma [174]. In some studies, IL-4 has been shown to induce apoptosis, and in others to protect from apoptotic death but to produce a cytostatic state [175–177]. Thus, its effect is complex and condition dependent.

Early clinical reports suggest varied effects from IL-4 but include growth inhibition of the lymphoma. Two phase I studies have demonstrated antitumor response, with shrinkage of lymph nodes in low-grade lymphoma and resolution of a lymphocytosis in CLL after IL-4 treatment [178,179]. We observed responses in CLL and low-grade lymphoma in an early pilot study as well (J. Dutcher and P. Wiernik, unpublished data). All these patients were heavily pretreated, so responses were brief. It is not possible to distinguish a cytostatic from a cytotoxic effect, but tumor shrinkage and reduction of CLL lymphocytes suggests some cytotoxicity. An ongoing study of IL-4 by the Eastern Cooperative Oncology Group (E5Y92) in less heavily treated patients may help to clarify this point.

We have reported one patient with a major response, and perhaps evidence of differentiation as well [180]. The patient, with B-cell chronic lymphocytic leukemia (CLL) producing a paraprotein and with plasmacytoid lymphocytes in the marrow, had complete resolution of lymphocytosis of marrow and peripheral blood after treatment with IL-4. However, subsequently, his B-cell disorder evolved into a combination of CLL and classical multiple myeloma, with lytic bone lesions, marrow plasmacytosis, and paraproteinemia. He responded to chemotherapy, but had residual CLL and myeloma cells in his marrow. With further chemotherapy, both have achieved complete remission.

IL-4 is also being utilized to induce activation of dendritic cells to generate immune responses. Activated dendritic cells are potent antigen presentors and are being used in vaccine studies in a variety of tumors, including lymphoma. IL-4 has yet to be utilized in conjunction with chemotherapy. The multitude of actions of IL-4 with respect to B-cell development make this an important cytokine to better understand, both as an endogenous growth factor and as an exogenous agent to induce antilymphoma responses.

Monoclonal antibodies and vaccines

B-cell malignancies have characteristic cell surface antigens that delineate the level of differentiation of the lymphoma, as well as the surface immunoglobulins that are specific to the malignant cells. These properties have generated great interest in the development of tumor-specific approaches such as activated monoclonal antibodies against B-cell antigens and idiotype-specific vaccines. Monoclonal antibodies directed at B-cell-specific antigens such as CD19 (B4), CD20 (B1), CD21, CD37, and others have been developed and refined and studied over the past decade [181–185]. Technical and immunologic issues were problematic early in the development of these agents, but recently effective antibodies — often conjugated with toxins [183,184] or radioisotopes [185–190] — have been reported to yield clinical responses. The conjugated agents include immunotoxins such as B4-blocked ricin [183,184] and radioisotopes such as ^{131}Iodine-Lym-1 [186,187], ^{131}Iodine-labeled LL2 [188], ^{131}Iodine-antiB1 [189–191], and ^{90}Yttrium anti-CD20 [192] — Kaminski et al., utilizing pretreatment with unlabeled antibody to saturate normal tissues, proceeded to treat patients with bulky lymphoma at a calculated dose of 25 cGy to the entire body [189,190]. In the pilot study, 6 of 9 patients had responses, including 4 complete and 2 partial responses, with one CR lasting 8 months and 3 patients stable at 8, 9, and 11 months [189]. An update of the use of this antibody was just published, in which 28 patients received a radioimmunotherapeutic dose [190]. All patients were heavily pretreated (median of three prior regimens), but 14 patients achieved complete response and eight a partial response [190]. Of interest, 6 of 8 patients with transformed lymphoma responded. Thirteen of 19 patients whose disease was resistant to their previous regimen responded to the radioimmunotherapy [190]. The median duration of complete remission is greater than 16.5 months, and six patients have remained in CR from 16 to 31 months. In addition, Press et al. reported high-dose radioimmunotherapy with bone marrow support in lymphoma, utilizing anti-CD20 and anti-CD37 antibodies labeled with ^{131}Iodine [191]. Of the 24 patients who had favorable biodistribution, 16 achieved complete remission and two partial, with one minor response. Nine patients remained in coninuous complete remission at 3–53 months at the time of the report [191]. Larger trials with these agents are ongoing.

Another approach pioneered by Levy et al. is the induction of an immune response to the idiotype immunoglobulin specific to the malignant B-cell clone [193,194]. A recent presentation and subsequent report of their work describes clinical responses to idiotype-specific vaccine therapy [195,196]. In this study, the vaccine is utilized to generate an idiotype-specific immune response following maximal response to autologous BMT. A significantly enhanced disease-free interval was reported compared to patients not receiving vaccine post-BMT [195]. Further investigation of these vaccines is ongoing. Others have investigated the anti-idiotype mechanism and have made radioimmunoconjugates with ^{90}Yttrium, and have reported clinical responses [197].

In summary, biotherapy in NHL appears to be an important clinical option, with clinically important outcomes being reported. Further refinement and development as we learn more about these agents and their interactions with lymphoma cells should yield continued improved results.

Future directions in biologic therapy in hematologic malignancies

As mentioned in the specific disease-oriented sections, there are a variety of new approaches called *biologic therapy* being investigated in hematologic malignancies. These include harnessing growth-regulatory molecules such as IL-1 and inhibitors of such molecules [34–37]. Despite the recognition of major biologic effects and immune activation, the exact mechanism of growth inhibition of agents such as IFNα, IL-2, and IL-4 remains controversial. Other biologic therapies that seem more directed toward immune modulation include monoclonal antibodies and vaccines that generate an immune response. Additional, less well-understood molecules such as bestatin and linomide demonstrate immune modulation in animal systems and may become clinically important.

Another drug that has a myriad of activities is cyclosporin A (CSA). We have already described the salutary effect observed in myeloma, presumably due to inhibition of production of the growth factor IL-6 [141,142]. However, CSA has many other effects, including as an immunosuppressive agent in allogeneic BMT [198,199]. Cyclosporin A inhibits the production of IL-2 in some settings but has also been shown to induce T-cell activation if certain cell surface receptors are activated [200,201]. This paradoxical effect has been studied in attempts to utilize CSA to induce graft-versus-leukemia in autologous BMT [202,203].

Another use of CSA in hematologic disorders has been the treatment of cytopenias that are presumed to have an autoimmune basis [204]. Cyclosporin A has been used successfully in refractory pure red cell aplasia secondary to CLL [205] and in the hemolytic anemia associated with CLL [206]. In the latter report, a salutary effect on the CLL itself was observed [206]. Cyclosporin A is also beneficial in some cases of severe idiopathic thrombocytopenic purpura [207]. We have reported dramatic benefit from CSA administration in ameliorating thrombocytopenia following treatment of AML in four patients who were all in remission with all cell lines present in the marrow, but with decreased megakaryocytes [208]. Despite its effect on T-cell activation, CSA has not been successful as a single agent in treating peripheral or cutaneous T-cell lymphoma [209]. Finally, the successful treatment of two cases of angioimmunoblastic lymphadenopathy with dysproteinemia, thought to be a disorder of T-cell regulation of B cells, has been reported [210]. The many aspects of the influence of cyclosporin A on the immune regulation of hematologic malignancies and disorders may be helpful in unraveling the defects inherent in the malignancies themselves, and may provide a better

understanding of mechanisms that in turn may lead to new therapeutic applications.

Summary

Biologic agents have made a major impact on the treatment of hematologic malignancies and will continue to play a major role as we better understand their function in normal and malignant cell regulation. The examples provided in this chapter are a brief introduction to the potential of these agents. Many have yet to be used in conjunction with current cytotoxic therapy in these diseases, and perhaps combinations will prove even more successful. This possibility provides substantial ground for further investigation and therapy.

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13. Recent advances in the biology and management of acute lymphoblastic leukemia in adults

Owen A. O'Connor and Mark Weiss

Acute lymphoblastic leukemia (ALL) is a malignant disease resulting from the unregulated clonal proliferation and arrested maturation of lymphoblasts. The phenotypic heterogeneity of the lymphoid neoplasia is a function of the complicated ontogeny these cells undergo in differentiation, as well as their multiplicity of activities in vivo. Evolving techniques in immunophenotyping and molecular genetics have refined our knowledge and classification of the lymphoproliferative diseases. These techniques allow lymphoid malignancies to be classified according to their specific lineage (B- or T-cell), their stage of differentiation (early pre-B, pre-B, or B-cell), and their genotype (specific translocations, oncogenes, etc.). Classifications of leukemia that rely exclusively upon morphology and histochemistry, such as the French–American–British (FAB) system, are limited by their inability to accurately stage the degree of differentiation or to identify molecular subsets of disease. These limitations do not allow adequate prognostication or risk stratification prior to the commencement of therapy. Knowledge of the lymphoblast molecular characteristics is becoming increasing important in defining new clinicopathologic correlations prior to commencing definitive therapy. Such is already the case with mature B-cell ALL (t(8;14)) and Philadelphia chromosome (t(9;22))-positive ALL.

ALL is the most common malignant disease of children [1]. Of the 3000 cases reported annually in the U.S., approximately one third will occur in adults, where ALL accounts for 15%–20% of acute leukemia [2]. Prior to modern chemotherapy, ALL was a uniformly fatal disease, with a median survival measured in weeks to months [3–5]. Current therapy cures approximately 60% to 80% of children with ALL, but only 20% to 40% of adult patients [4,6]. These striking differences in treatment response, as well as new information regarding the clinical outcome of different molecular classes of patients, supports the theory that childhood and adult ALL represent two different subsets of the disease.

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Epidemiology and etiology

The leukemias are infrequent diseases, accounting for 3% of all cancers in the U.S. In the U.S., it is estimated that there will be approximately 28,300 cases of leukemia in 1997, of which about 10% will be ALL [2]. As with lymphoma and leukemia in general, there is a slight made predominance (53%). The expected annual mortality from all leukemias in 1997 is anticipated to be approximately 21,300 [2]. Thus, the case-fatality rate for leukemia in general (i.e., the number of deaths attributed to the disease/the incidence of the disease) is approximately 70%. The case-fatality rate for ALL is about 47%, while the other leukemias have rates that range from 56% to greater than 75% [2]. This divergent statistic for ALL is attributed to a feature of this disease that distinguishes it from other hematologic malignancies: It is the malignancy of childhood, which, in children, is highly curable. The peak incidence of ALL occurs at approximately 4 years of age, with a second smaller peak at 70 years of age [7,8].

The strongest evidence for a link between an environmental exposure and leukemia comes from Japanese survivors following the detonation of the atomic bombs at Hiroshima and Nagasaki in 1945. Over 93,000 survivors of the event were followed through the efforts of the Radiation Effects Research Foundation. These data revealed that the earliest malignancy to appear following radiation exposure was leukemia, which became apparent approximately three years following the detonation, with a peak incidence at about eight years. The most important factor influencing the risk of leukemia following detonation of the A-bomb was age at the time of the explosion. The younger the individual, the shorter the latency period. The average latency period for all forms of leukemia was five years for children under the age of 15, compared to 20 years for individuals 45 years and older [9]. These data demonstrate a linear relationship over the age groups studied. Interestingly, the patterns of leukemogenesis were distinctly different between Nagasaki and Hiroshima. In Nagasaki, essentially all cases of leukemia were AML. In Hiroshima, a greater diversity of leukemias were found, including AML, CML, and ALL [10]. The plutonium device used at Hiroshima yielded both γ-rays and neutrons, while the plutonium bomb used at Nagasaki produced only γ-rays [11]. Over the years, the dosimetry data collected has been reanalyzed, with several readjustments of the relative biological effectiveness (RBE) of γ-rays and neutrons in radiation carcinogenesis. Nonetheless, some investigators have interpreted these data to suggest that CML and ALL are more neutron-dependent leukemias, whereas AML may be more γ-radiation dependent.

Despite the data from Japanese survivors of the atomic bomb, there is still an enormous controversy surrounding the leukemogenic effects of radiation [12]. Unlike the survivors of the atomic bomb, who received a single large dose of radiation (100 cGy), most individuals are exposed to smaller multiple fractions of radiation. Most cumulative data to date suggest that hyperfractionated

radiation in the range of 1 to 300 rem (the amount of radiation that produces a biological equivalent resulting from 1 cGy of γ-rays) is weakly, if at all, leukemogenic [12,13].

The best known association between a discrete chemical exposure and leukemia has been documented with benzene [14–18]. Several epidemiological studies performed on workers exposed to high concentrations of benzene through their occupation have demonstrated an excess of leukemia in the exposed workers. In one such study [19], 33 cases of leukemia were reported in over 28,000 shoe workers chronically exposed to benzene over a seven-year period. Overall, there was a twofold increase in AML but no increase in ALL. Though some studies report an occasional cases of ALL in benzene-exposed individuals, most studies suggest that the risk of ALL is negligible [14,20].

One of the most publicized associations regarding ALL and an environmental risk factor came to public attention in the late 1970s, when residential power lines and radio towers were indicted as a cause of childhood ALL. At best, these early studies were inconsistent [21–24]. Subsequently, several studies have demonstrated an excess of leukemia among children exposed to residential magnetic fields. These studies, in general, are limited by their reliance on surrogates of magnetic field exposure [21,25]. Since there is no scientifically validated biomarker of exposure to electromotive forces (EMF), many studies depend on nonquantitative surrogates of exposure, such as visual inspection of neighboring powerlines and known wire-coding. In a recently reported study, Linet et al. [26] enrolled 638 children diagnosed with ALL, as well as a control cohort, into a study that not only assessed local wire coding but also measured magnetic fields in the homes of all children. These data did not support a relationship between high time-weighted average magnetic field levels and the risk of childhood ALL. Furthermore, the study could not identify a relationship between the highest wire-code category and childhood ALL, in contrast to other published reports.

An increased incidence of ALL has been reported in children diagnosed with Down's syndrome [27], Bloom syndrome [28], Fanconi's anemia [29], and ataxia telangiectasia [30]. In addition, it appears there is an increased risk of ALL in siblings of patients who have been diagnosed with ALL [31,32]. While the nature of these associations is at present unclear, some evidence suggests that hereditary or acquired mutations in p53 may account for some cases of childhood ALL [33].

Classification and characterization

ALL is a heterogeneous disease. Morphology and histochemistry are the initial means by which the disease is diagnosed, and they assist in differentiating lymphoid from myeloid leukemias. In general, however, state-of-the-art diagnosis requires immunophenotypic and cytogenetic characterizations for defining clinically important prognostic subsets.

Morphology

Morphologic classification of ALL is based upon the French–American–British (FAB) Cooperative Working Group criteria presented in Table 1 [34]. This classification scheme divides lymphoblasts into one of three distinct morphological categories, namely, L1, L2, and L3 (Burkitt's type). In the L1 subtype, the lymphoblasts are uniformly small, with scant mildly basophilic agranular cytoplasm, containing round nuclei with an inconspicuous nucleolus. Subtype L1 ALL typically constitutes 85% of childhood and 30% of adult ALL [34,35]. The L2 subtype is characterized by more heterogeneous lymphoblasts that are generally larger and contain more abundant cytoplasm with conspicuous nucleoli. This subtype constitutes the majority of adult ALL (about 70%) and only 10%–15% of childhood cases [34,35]. Previously, this subtype was thought to have an adverse prognosis, but with modern therapy there is little prognostic significance associated with the L1 versus L2 distinction. The L3 subtype, or Burkitt's variant, comprises less than 5% of ALL cases and is characterized by large pleomorphic lymphoblasts, with deep blue cytoplasm and prominent cytoplasmic vacuoles.

Immunophenotyping

The biological basis for understanding the diversity of lymphoid neoplasia is best understood in the context of lymphocyte ontogeny (Figures 1 and 2). The maturation of lymphoblasts proceeds through a series of developmental stages characterized by changes in the presence or absence of various cell surface molecules (identified as clusters of differentiation [CD] markers), terminal deoxynucleotidyl transferase (TdT; a DNA polymerase), as well as cytoplasmic and surface immunoglobulin. At present, over 100 different CD antigens have been identified on the surfaces of hematopoietc cells. These antigens are routinely expressed on the surface of different hematopoietc cells, and their specific patterns of expression forms the basis for immunophenotyping.

Table 1. French–American–British (FAB) classification of acute lymphoblastic leukemia

Morphology	L1	L2	L3
Cellular size	Small homogeneous cells	Larger heterogeneous cells	Large homogeneous cells
Cytoplasm	Scantly with mild basophilia	More abundant with more basophilia	Abundant with intense basophilia
Nucleus	Round with occasional clefts	Irregular and frequently clefted	Round-to-oval without clefts
Nucleoli	Usually not apparent	Often present	Multiple and prominent
Vacuoles	Variable	Variable	Abundant
% Pediatric cases	85%	10%–15%	1%–2%
% Adult cases	25%–30%	70%	1%–2%

In comparison to normal lymphocyte differentiation and maturation, the pattern of CD antigen expression on malignant cells is often aberrant [36]. In some cases, there is also abnormal expression of antigens not expected to be found in the lineage [37–40]. Although such 'infidelity' of antigen expression

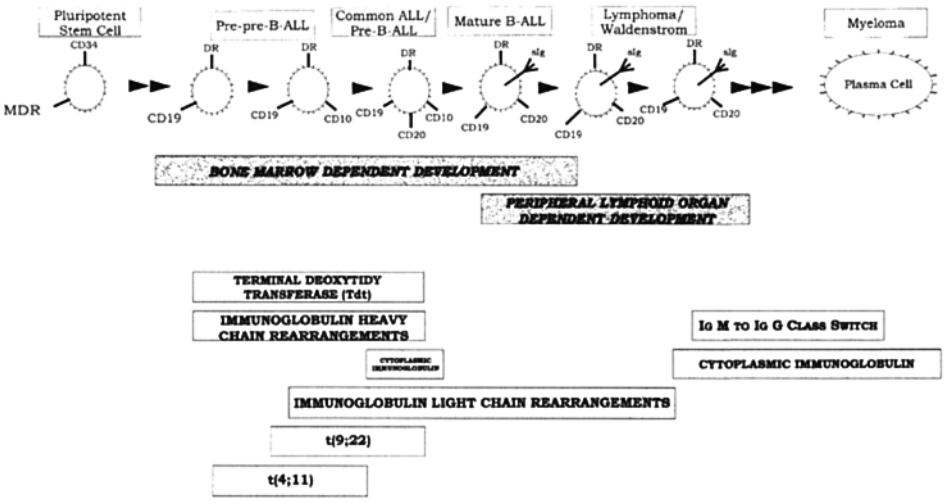

Figure 1. Schematic of B-cell lymphocyte ontogeny with specific reference to immunophenotypic and cytogenetic changes as a function of maturation, as well as the relationships to other lymphoproliferative disorders.

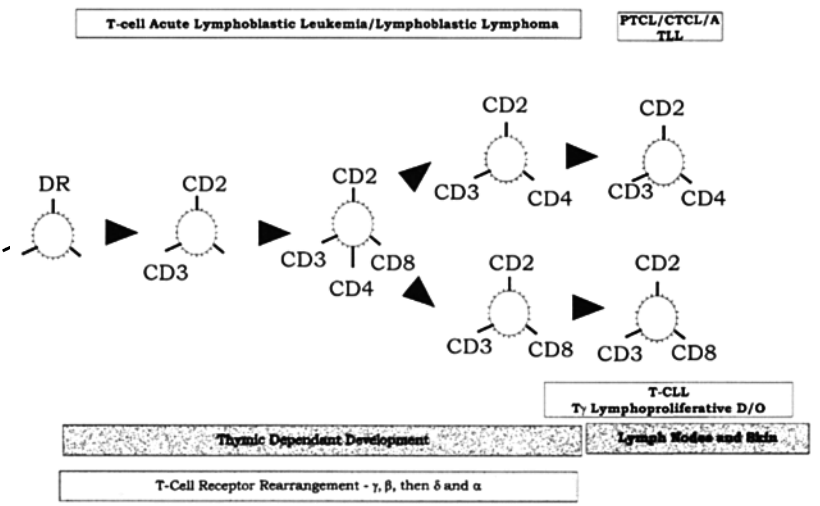

Figure 2. Schematic of T-cell lymphocyte ontogeny with specific reference to immunophenotypic and cytogenetic changes as a function of maturation, as well as the relationships to other lymphoproliferative disorders.

may prevent exact assignment of the maturational stage of the leukemic cell, it may distinguish that cell as neoplastic from within a larger population of normal cells. Thus, aberrant antigen expression, which can include antigens of the wrong lineage, the simultaneous expression of antigens of different stages of maturation, or the lack of an expected antigen, can provide a useful diagnostic marker for the leukemic cells [37]. In addition, such a marker may allow the flow cytometric detection of the leukemic cells at very low levels of residual disease. One report suggests that this test may be as sensitive as detecting 1 leukemic cell in 100,000 normal cells [41].

Approximately 80% of all ALL cases are of B-cell lineage. B-cell lineage ALL is subclassified, based upon its stage of differentiation, into early pre-B-cell, pre-B-cell, and mature B-cell ALL. The remaining 20% of ALL cases are of T-cell origin. T lymphoblasts in patients with T-cell ALL appear to be arrested in early intrathymic stages of maturation and are distinct from other T-cell neoplasms such as peripheral T-cell lymphoma (PTCL), cutaneous T-cell lymphoma (CTCL), and adult T-cell leukemia/lymphoma (ATCL). Figure 2 details the immunophenotypic characteristics of these different subsets of T-cell ALL.

ALL that includes very immature B-lineage lymphoblasts (sometimes referred to as pre-pre-B ALL or early pre-B ALL) expresses TdT, HLA-DR, and CD19 and displays rearrangements of both immunoglobulin heavy and immunoglobulin light chains [42–44]. It lacks cytoplasmic or surface immunoglobulin and CD10, formerly referred to as the common ALL antigen (CALLA). This pre-pre-B ALL phenotype is commonly associated with the t(4;11), which typically presents with a marked leukocytosis. Because of the associated karyotype, pre-pre-B ALL has a poor prognosis. This particular subset of ALL typically occurs in 11% of adults and about 5% of children [45–47].

The most common ALL immunophenotype (pre-B) is similar to pre-pre-B ALL, except it also expresses the cell surface antigen CD10 and cytoplasmic immunoglobulin. These cells are termed *pre-B* because they lack the hallmark of the mature B cell, namely, surface immunoglobulin. This variant, frequently referred to as *common* ALL, constitutes approximately 65% of childhood ALL and 50% of adult ALL [41–43].

Mature B-cell ALL expresses surface immunoglobulin along with CD19 and CD20. Though their normal counterparts do not, these cells may also express CD10 and/or TdT. This disease accounts for less than 5% of all ALL [42–44].

T cells also undergo serial steps of differentiation, differing from B cells in that their differentiation occurs in the thymus. Maturation of these cells is typically divided into three stages. Stage I and II thymocytes reside in the cortex of the thymus, while stage III thymocytes reside in the thymic medulla. Initially, T lymphoblasts are positive for TdT and express HLA-DR. The CD2 (the E rosette receptor), CD5, and CD7 (gp40) antigens can be detected

throughout all stages of normal T-lymphoblast ontogeny [42–44]. The mature T-cell antigens CD4 and CD8 can be present on lymphoblasts but are coexpressed only on stage II thymocytes. Further maturation leads to loss of either CD4 or CD8 (stage III thymocytes), with the resulting mature T-cell phenotype of the T-helper (CD4+/CD8−) or T-suppressor (CD4−/CD8+) cells.

Typically, increased maturation of the T lymphoblast is associated with increased rearrangements of the T-cell receptor (TCR) genes. Four genes code for each of the peptides that constitute the TCR and are referred to as α, β, γ, and δ. Any given T cell will express only one type of TCR, either αβ or γδ. At a molecular level, the differentiation of the TCR undergoes sequential rearrangements, such that the genes encoding for the γ and β peptides are the first to undergo rearrangement, followed by the δ gene and α genes, respectively [48]. This molecular differentiation of the T lymphoblast has significantly refined our ability to assign these cells to discrete stages of development. While most T-cell ALLs have undergone one or more rearrangements of the TCR, some investigators have proposed a classification similar to that employed for B-cells above. Early T-cell ALL (or, pre-T ALL) would consist of cells with one or two rearrangements of the TCR. More mature T-cell ALL would be characterized by rearrangements of the δ and α genes [48].

Malignancies derived from the relatively immature intrathymic thymocytes are termed *lymphoblastic lymphoma/T-cell ALL*. Malignancies derived from mature, postthymic T cells include a variety of lymphoproliferative diseases such as peripheral T-cell lymphoma (PTCL), cutaneous T-cell lymphoma (CTCL), adult T-cell leukemia/lymphoma, and Tγ lymphoproliferative disorders. Adult T-cell leukemia/lymphoma is strongly associated with HTLV-1 and is relatively uncommon in the U.S. The disease is most commonly found in regions where HTLV-1 is endemic, such as Japan and the Caribbean.

Cytogenetic characterization

The identification of multiple nonrandom chromosomal translocations has contributed significantly to our understanding of leukemia pathogenesis. Correlation of particular genetic lesions with clinical behavior and response to treatment has provided a framework for rational ALL classification.

Over 50% of all ALL cases carry chromosomal translocations [49,50]. There are a large number of specific cytogenetic lesions identified to date, and this number continues to increase rapidly [51–57]. In general, two mechanisms of oncogenic conversion have been recognized in ALL. In the first, juxtaposition of a silent or low-expression proto-oncogene close to a high-activity promoter leads to deregulation of the proto-oncogene, with consequent gene overexpression [53–56]. Such is the case in the Burkitt's translocation (t(8;14)),

when c-*myc* on chromosome 8 is brought into close proximity to the promoter of the immunoglobulin heavy chain gene on chromosome 14 [58,59]. In the second example, interchromosomal recombination allows the production of a fusion transcript resulting in a chimeric protein. These chimeric proteins possess new (or unregulated) biological activity accounting for the malignant phenotype. This mechanism is seen in the Philadelphia chromosome (t(9;22)), which fuses *bcr* (breakpoint cluster regions) on chromosome 22 to the proto-oncogene *abl* on chromsome 9 [60–63].

Many cytogenetic abnormalities have prognostic value in ALL, including the adverse prognosis associated with hypoploidy, t(4;11)(q21,q23), and t(9;22)(q34;q11) [64–66]. Favorable prognoses have been documented for hyperdiploidy (50 or more chromosomes) and t(12;21), which produces a tel-AML-1 fusion product [67]. Previously, the Burkitt's translocations, namely, t(8;14)(q24;q32), t(2;8)(p11;24), and t(8;220(q24;q11), were viewed as adverse prognostic features. Recently, regimens developed specifically for this disease have dramatically improved treatment results for this entity.

Drug resistance

Emerging data have begun to correlate clinical outcome with specific markers of drug resistance. The ability of lymphoblasts from adult and pediatric patients with ALL to form long-chain methotrexate polyglutamates has been shown to correlate with treatment outcome [68,69]. Polyglutamylation of methotrexate leads to increased intracellular accumulation of the drug and thus greater cytotoxic activity. Both T-lineage ALL blasts from children and adults and B-lineage blasts from adults accumulate lower concentrations of long-chain polyglutamates of methotrexate than B-lineage blasts from children [70,71]. Those children with pre-B-cell ALL who accumulated high concentrations of methotrexate polyglutamates had a longer event-free survival than patients whose blasts accumulated only low concentrations of methotrexate. Interestingly, hyperdiploid B-lineage lymphoblasts (a good prognostic feature) accumulated higher concentrations of long-chain polyglutamates compared to euploid or hypoploid lymphoblasts [72]. It is hypothesized that this increased sensitivity to methotrexate may be the mechanism contributing to the favorable prognosis of hyperdiploid lymphoblasts.

In addition, lymphoblasts from adults with ALL demonstrate a significantly higher degree of in vitro corticosteroid resistance when compared to pediatric cases. While the mechanisms of this resistance are not yet clear, overexpression of transmembrane transporter molecules, including P-glycoprotein (the multidrug-resistance-associated protein) may be an important mechanism of drug resistance [73]. Expression of P-glycoprotein has been associated with a lower probability of achieving a remission with induction chemotherapy and has been associated with a higher relapse rate in adult ALL [74].

Clinical manifestations

The clinical presentation of patients with ALL primarily results from soft tissue infiltration by lymphoblasts and the consequences of bone marrow failure (Table 2). Although the signs and symptoms of ALL are relatively nonspecific, a thorough history and physical examination, in conjunction with a review of the peripheral blood smear and bone marrow aspirate, are obligatory in the diagnostic evaluation. Physical exam findings such as pallor, tachycardia, and tachypnea are the hallmarks of anemia, while petechiae, easy bruisability, epistaxis, or gingival bleeding are typical for thrombocytopenia. The stigmata of infection, including fever, chills, and diaphoresis, are commonly present. In the setting of absolute neutropenia, these findings are almost always secondary to active infection and require prompt medical attention. In one report of adult patients presenting with ALL, approximately one third of patients had fever and/or hemorrhage [75].

Direct tissue infiltration by lymphoblasts produces lymphadenopathy, splenomegaly, and or hepatomegaly in approximately 50% of patients at diagnosis. Tissue infiltration can occur in any subtype of ALL but is most common in T-cell and mature B-cell disease. In T-cell disease, symptoms attributable to a mediastinal mass and pleural or pericardial effusion are common. In mature B-cell (Burkitt's) ALL, abdominal, testicular, and CNS disease may frequently be seen at presentation.

The laboratory evaluation of patients with ALL typically reveals anemia and thrombocytopenia. Severe thrombocytopenia (less than 50,000/μL) occurs in more than one half of all patients. The total white blood cell (WBC) count

Table 2. Typical diagnostic findings of acute leukemia in adults

Symptoms	
• Infection related	Chills, diaphoresis
• Bleeding related	Epistaxis, easy bruisability, gingival bleeding, hemoatochezia, melena, hematuria
• Anemia/nonspecific/constitutional	Fatigue, malaise, dyspnea, weightloss, neurologic complaints including headache
Signs	
• Infection related	Fever, pneumonia, perirectal abcess, sepsis
• Bleeding/anemia related	Pallor, ecchymoses, petechiae, fundal hemorrhage, tachycardia
• Tissue infiltration related	Lymphadenopathy, splenomegaly, hepatomegaly, mediastinal mass, focal neurologic findings
Laboratory and diagnostic evaluation	
• Diagnostic	CBC and differential, review of peripheral blood smear, bone marrow aspiration and biopsy, immunophenotyping, chest x-ray and directed CT scanning, lumbar puncture with cytology
• Prognostic	Cytogenetics
• End-organ function/pretreatment evaluation	Serum chemistries, coagulation studies, echocardiography

can be normal, elevated, or reduced; approximately 10%–20% of patients have WBC counts greater than 100,000 cells/μL, while approximately 25%–30% will have WBC counts less than 5000 cell/μL [35]. Examination of the peripheral blood smear reveals blasts in the majority of cases. Minor coagulopathy is common, and most patients present with some elevation in the prothrombin and partial thrombopastin times (PT/PTT). More severe coagulopathies (i.e., fibrinogen <100 mg/dL) are infrequently seen. In comparison to acute promyelocytic leukemia (APL), clinically important disseminated intravascular coagulation is a relatively rare finding at presentation in adult patients with ALL but may be precipitated by treatment [76].

Serum blood chemistries are typically within normal limits, with the exception of lactate dehyrogenase (LDH), which is frequently elevated, particularly in mature B-cell ALL. Tumor lysis syndrome is perhaps the most common serum chemistry abnormality seen in ALL, and is particularly associated with the Burkitt's subtype. It is classically observed during the initial treatment phase, and if not properly anticipated and treated can lead to fatal arrhythmias and/or acute renal failure. The syndrome is characterized by hyperkalemia (secondary to release of intracellular potassium), hyperphosphatemia (secondary to increased DNA degradation), hypocalcemia (secondary to protein and phosphate complex formation), and hyperuricemia (secondary to increased purine catabolism from lymphoblast DNA) [77].

A routine chest x-ray should be obtained on all patients to assess for the presence of a mediastinal mass and preexisting pneumonia and to provide a baseline examination prior to the institution of therapy. Computed axial tomography (CT scan) should be performed for patients with T-cell and mature B-cell disease, and as directed by symptoms and physical exam findings in patients with pre-B-cell ALL.

Approximately 10% of patients will have demonstrable CNS disease at diagnosis [35]. Approximately one half of these patients will have symptoms of CNS involvement including headache or (more commonly) cranial nerve palsy, while the other half will be completely asymptomatic. A lumbar puncture should be performed on most patients at presentation, with two main exceptions. The first is patients with significant thrombocytopenia (platelets <50,000/μL) or coagulopathy, where the risk of epidural hemorrhage with possible resultant paralysis may be increased. The second is patients with a significant number of circulating lymphoblasts. In this setting, a minimally traumatic lumbar puncture may inadvertently contaminate the cerebral spinal fluid (CSF), leading to an incorrect diagnosis of leukemic meningitis. Though not substantiated in the literature, another area of concern is the theoretical risk of seeding the CSF with lymphoblasts during a traumatic lumbar puncture, leading to the development of leukemic meningitis. These two contraindications, however, are only relative, and patients with signs or symptoms of leukemic meningitis should have their coagulopathies (if present) corrected and a diagnostic lumbar puncture performed. Since all patients with ALL will require intrathecal (IT) prophylaxis, it is reasonable to instill

intrathecal methotrexate at the initial lumbar puncture (see Treatment section below on CNS disease management and prophylaxis). A noncontrast CT is adequate for evaluating patients for cerebral hemorrhage or signs of herniation, but a contrast MRI of the brain is required to detect meningeal leukemia.

Prognostic features of adult ALL

Five adverse prognostic risk factors have been identified through multivariate analysis in adult ALL (Table 3). These include elevated white blood cell count, B-cell immunophenocyte, older age, presence of the Philadelphia chromosome (Ph+), and longer time to complete remission.

Leukocytosis at diagnosis is associated with a poor prognosis [78,79]. There is a reduced likelihood of achieving a complete remission (CR), as well as a shortened duration of remission (and overall survival) for those who achieve a CR. The significance of leukocytosis may be a function of its association with adverse cytogenetics such as the t(4;11) and t(9;22), as well as a marker of leukemic burden and behavior. Some studies have suggested different thresholds above which the WBC indicates a poor prognosis. The analysis performed at Memorial Sloan Kettering Cancer Center (MSKCC) indicates that a WBC count greater than 10,000/μL was associated with a lower probability of CR, while a WBC count greater than 20,000/μL was associated with a shorter duration of CR [79]. The German analysis suggested a cutoff at 30,000/μL. It seems likely that an elevated WBC count is a continuous prognostic variable in adult ALL [78].

Age is one of the most striking prognostic variables in ALL. Older age is associated with a worse prognosis [78–80]. In the pediatric literature, adolescent patients typically have the poorest prognosis, compared to children less than age 8. In the adult literature, adolescents have the better prognosis, while older patients have the poorest prognosis. Age is almost certainly a continuous

Table 3. Prognostic features of adult acute lymphoblastic leukemia

Prognostic feature	Influence on outcome
Age	The greater the age, the worse the prognosis.
White blood cell (WBC) count	The greater the WBC, the worse the prognosis. WBC count is likely a continuous variable.
Immunophenotype	T-cell is most favorable prognosis, pre-B-cell has an intermediate prognosis, and mature B-cell disease has the worse prognosis with standard regimens.
Cytogenetics	Translocations involving t(9;22) (Ph+) and t(4;11) are associated with a worse prognosis.
Time to complete remission (CR)	Patients requiring more than 4–5 weeks to attain a CR have a poor prognosis.

variable (the older the age at diagnosis, the poorer the prognosis), with the two most commonly recognized thresholds being age 35 (German study) and age 60 (MSKCC study) [78,79].

The immunophenotype of the leukemic cell carries prognostic significance [78,79,81]. With current treatment regimens, T-cell disease has the most favorable prognosis, while pre-B-cell ALL has a somewhat worse prognosis. With standard ALL therapy, mature B-cell disease had a very poor prognosis. However, recently described regimens designed specifically for this entity have led to dramatically improved results (see Mature B-cell ALL section below). Historically, T-cell ALL was associated with a poor prognosis. The improvements in disease-free survival noted over the last 10 to 15 years for T-cell disease have been primarily attributed to the incorporation of cytarabine and cyclophosphamide into the treatment regimen [83,84]. Previously, the expression of a single myeloid antigen (typically CD13 or CD33) on the lymphoblast (My+) was thought to be an adverse prognostic feature. Larger studies using modern regimens indicate that this entity does not have a poor prognosis [35].

The Philadelphia chromosome carries a very poor prognosis [78,79]. Only about 4% of children are Ph+, in comparison to 40% to 50% of adults over age 50 with B-lineage disease (t(9;22) is not found in T-cell disease). Adult patients with this cytogenetic abnormality are essentially never cured by standard chemotherapy, and in general, the few patients who are cured are those who undergo allogeneic bone marrow transplantation [86]. Another cytogenetic lesion associated with a poor prognosis is t(4;11). This lesion, involving the mixed lineage leukemia (MLL) gene at 11q23, may account for 10% of adults with B-lineage ALL [45,46].

The time required to achieve complete remission is inversely related to remission duration. Patients who require more than four weeks (German study) for five weeks (MSKCC study) to achieve a CR have a lower probability of cure [78,79]. The disease-free survival for patients who attained an early CR was approximately 42% to 46%, compared with 0% to 26% for those requiring additional treatment.

Other adverse prognostic features that have been discussed in the literature include FAB morphology, degree of bone marrow involvement, weight loss, LDH levels, and SGOT. In general, however, multivariate analysis does not support the clinical utility of these risk factors.

Treatment

A variety of different treatment regimens for adult ALL have been developed over the last 25 years. These regimens, which have been modeled on treatment programs developed for children, have been far less successful in adults [87–94]. Some of the difference in overall survival can be attributed to differences in ability to tolerate intensive chemotherapy. In addition, there is an increased

incidence of unfavorable cytogenetic subgroups in adults (especially the Ph+ ALL and t(4;11)) and a marked decreased incidence of favorable cytogenetic subgroups in the adult (in particular, hyperdiploidy and t(12;21)) [80].

Treatment of adult ALL can be divided into four broad categories: induction, consolidation, maintenance, and CNS prophylaxis. At presentation, many patients are quite ill with active infection and/or hemorrhage. This situation has led to the development of induction regimens that emphasize relatively myeloid-sparing cytotoxic agents. In contrast, once patients achieve a CR with recovery of normal blood counts and performance status, they are more likely to tolerate the toxicity associated with myelosuppressive agents. Consolidation therapy is therefore administered at a relatively high level of intensity to patients who achieve a complete remission. Figure 3 presents a schematic of a typical adult ALL treatment regimen (L-20).

Maintenance therapy is administered to patients in remission following the more intensive consolidation therapy. It is administered at a low level of intensity for a protracted period of time. While limited data exist to support a definitive duration of maintenance, most treatment regimens use a minimum of two years of maintenance therapy.

CNS prophylaxis is required to minimize the risk of developing leukemic meningitis. Despite otherwise aggressive systemic treatment, the CNS remains a sanctuary site, and without specific meningeal-directed therapy, approximately 35% of adult patients develop frank CNS disease [95].

Primary treatment for newly diagnosed adult ALL

The goal of any induction regimen is to achieve a CR — that is, complete eradication of the disease on morphological reevaluation, with consequent reconstitution of normal hematopoiesis. Present therapies successfully induce CR in approximately 65%–85% of unselected adults [87–96]. Unfortunately, most of these patients subsequently relapse, and overall, only 20%–30% of adults with ALL with be cured of their disease [87–96]. The mainstay of induction therapy for ALL has been the combination of vincristine and prednisone, which alone can achieve a CR in 50% of patients, with a median remission duration of 3–7 months [96]. Addition of an anthracycline to induction therapy was demonstrated in a randomized trial to increase the likelihood of achieving a CR from 47% to 83% [97]. This result has led to the incorporation of an anthracycline into all current adult ALL induction regimens. Interestingly, the increased incidence of CRs did not translate into improved survival in this study.

Although not supported by randomized trials, further intensification of induction chemotherapy with cyclophosphamide and/or L-asparaginase is widely accepted as improving remission induction [35]. Thus, one or both of these drugs are included in essentially all induction regimens. Present induction regimens are therefore defined as *four-drug* (vincristine, prednisone, anthracycline, and cyclophosphamide or L-asparaginase), or *five-drug*

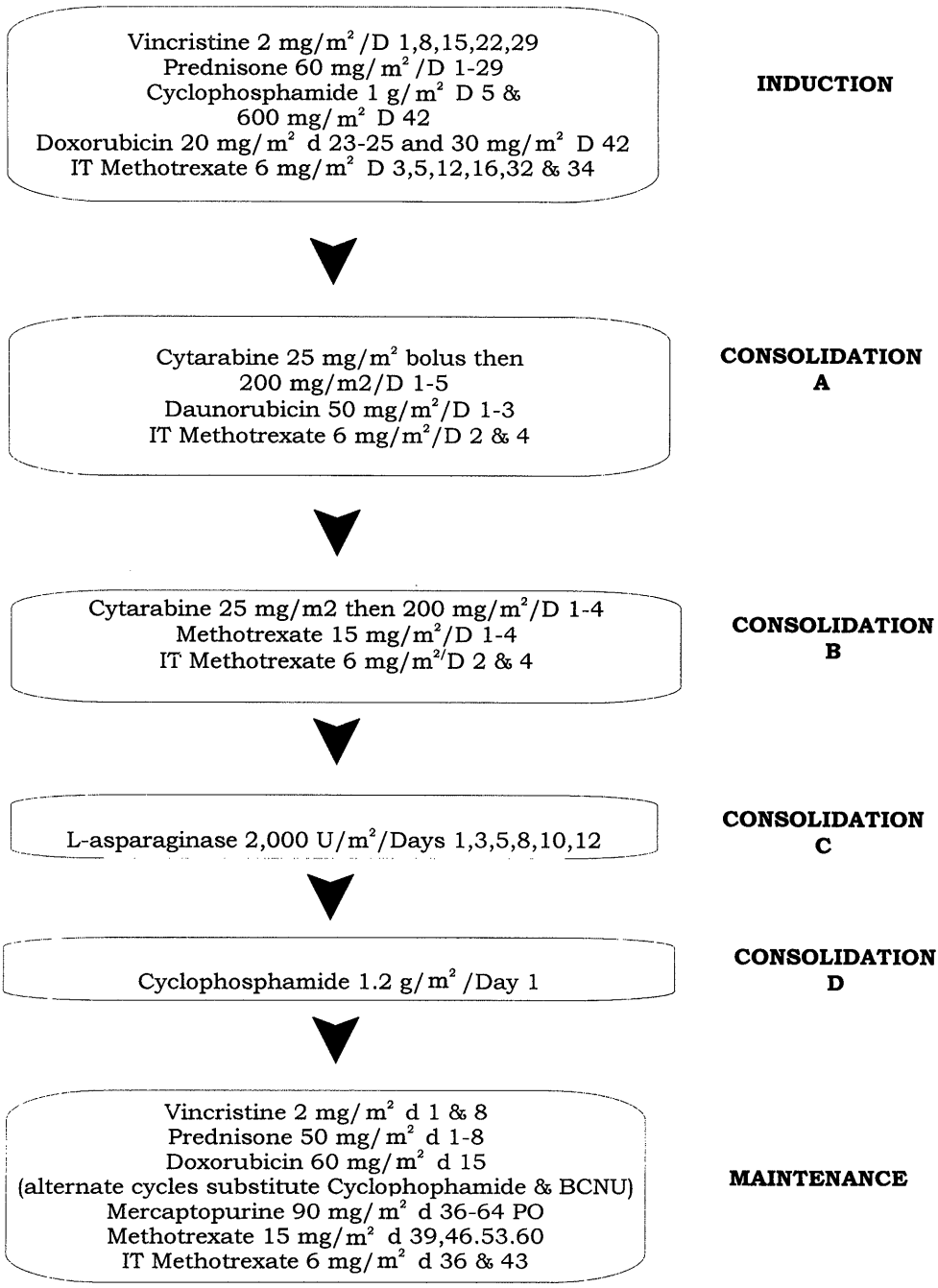

Figure 3. Schematic of a typical treatment regimen (L-20) used in the management of adult ALL.

regimens (vincristine, prednisone, anthracycline, cyclophosphamide, and L-asparaginase). There is no evidence to indicate that one treatment regimen is superior to another [35].

Consolidation therapy has evolved over time to include several drugs given in varying sequence. Although there is no standard consolidation therapy, some generalizations can be made. The drug most prominently employed is cytarabine (Ara-C). Consolidation regimens include cytarabine in combination with anthracyclines, epidophylotoxins, or antimetabolites (such as methotrexate or mercaptopurine). Multiple studies support the value of consolidation therapy in adult ALL. Most of these reports however, are uncontrolled phase II studies or comparisons with historical controls [88,91,93,94]. In one such study at M.D. Anderson, three-year survival increased from 15% to 40% following the addition of consolidation therapy to the treatment regimen [98]. A randomized phase III trial demonstrating the importance of cytarabine-containing consolidations was reported by Fiere and colleagues [87]. In this study, following remission induction, patients were randomized to receive consolidation therapy with cytarabine, doxorubicin, and asparaginase, or to immediately receive maintenance therapy. Three-year survival in the consolidation arm was markedly superior at 38% compared to 0% in the no-consolidation arm ($p < 0.005$).

Protracted maintenance therapy is a feature unique to the management of ALL. All other human malignancies that are cured with chemotherapy require 3 to 6 months to complete the necessary treatment. In pediatric ALL, maintenance therapy of 2 to 3 years duration is required for optimal results. In adults, the necessity of maintenance therapy is less well established. To date, very few studies have attempted to omit maintenance therapy. At least two trials that did not use maintenance chemotherapy have been reported. Both the CALGB and the ECOG trials reported a lower incidence of disease-free survival (18% at three years and 13% at four years, respectively) than is seen with regimens that include maintenance therapy [9,100]. These results support the use of maintenance therapy in adult ALL. In another recently published study, intensive postremission consolidation chemotherapy after standard induction, without any maintenance therapy, yielded markedly reduced overall survival and disease-free survival in adults with ALL [101]. While there is no direct comparison of maintenance versus no-maintenance therapy in adult ALL, increasing evidence substantiates the critical role of maintenance therapy. The most important drugs used in maintenance therapy are a combination of oral methotrexate and mercaptopurine. In pediatric ALL, these two drugs alone may be sufficient. However, given the poorer prognosis in adults, more intensified maintenance programs including vincristine, prednisone, anthracyclines, and/or cyclophosphamide are usually employed.

The treatment of sanctuary sites is an essential feature of ALL management in both adults and children. The CNS, eye, and testis are the classic sanctuary sites, and represent tissues where the distribution of systemically administered cytotoxic therapy is inadequate. The insufficient penetration of chemotherapy

into sanctuary sites leads to the potential for relapse in these locations. Of the three sanctuary sites, the CNS is by far the most commonly affected. Typically 5% to 10% of adult patients have CNS disease at presentation [95,102]. The risk of CNS involvement is increased with elevated white blood cell counts and with mature B-cell disease [103,104]. Patients who do not receive specific CNS-directed prophylactic therapy have a cumulative risk of CNS relapse of approximately 35%, compared to 10% for those receiving such therapy [95]. While intrathecal therapy can be administered by lumbar puncture or intraventricularly via Ommaya reservoir, the latter is considered the preferred route, since treatment of the entire CNS is superior with this approach. Administration of intra-Omaya methotrexate permits equivalent CNS prophylaxis without the need for whole-brain radiation therapy (WBRT) [35]. In general, it is recommended that patients have their Ommaya reservoir placed following the attainment of a CR, in order to avoid performing the procedure in those patients who die during induction chemotherapy or who are identified as having primary refractory disease. Though a variety of intrathecal schedules have been utilized, a schedule employed at MSKCC wherein WBRT is not administered delivers six intrathecal methotrexate treatments (6 mg/m^2 with a maximum single dose of 15 mg) during the first two months of therapy [35]. Subsequently, patients receive two doses per month during consolidation and two doses every three months during maintenance therapy. The addition of WBRT to intrathecal therapy reduces the number of methotrexate doses required and allows intrathecal therapy to be administered via lumbar puncture, obviating the need for an Ommaya reservoir. Increasing concerns regarding late cognitive deficits, however, have led some physicians to favor approaches that omit radiation therapy from the treatment plan. Presentation (or relapse) with ocular or testicular disease in the adult is unusual, but when it occurs it is often in the setting of a mature B-cell ALL or Ph+ ALL. Failure in the eye or testis requires treatment with local radiotherapy as well as systemic chemotherapy.

The comparison of results of different treatment regimens in ALL is a complicated undertaking. As discussed, there are many prognostic factors that influence outcome in ALL. These include age, leukocytosis, immunophenotype, cytogenetics (in particular the Philadelphia chromosome), and time to complete remission. Normalizing results of reported clinical trials for each of these factors, allowing for a 'clean' comparison, is difficult, if not impossible. Over the last 20 years, treatment regimens have evaluated many formulations of the basic strategy outlined above. These variations have included drug type and dose, induction schedule, intensity of consolidation, and duration of maintenance. While a substantial experience has evolved, no single treatment regimen has proved to be superior.

Another problem is that treatment results are reported at various durations of follow-up. Experience with 272 adult patients treated for adult ALL at MSKCC, according to the principles described above, reveals that the survival curve does not plateau until six years from diagnosis. While 50% of patients

were alive at two years, this number decreased to 24% at six years and beyond [35]. Therefore, studies with short follow-up (i.e., 2 to 3 years) can markedly overestimate their ultimate results.

Treatment of relapsed or refractory disease

Conventional induction regimens for adult ALL obtain complete remissions in 65% to 85% of patients with newly diagnosed disease. Early deaths occur in 5% to 10% of cases, and 10% to 25% of patients will have disease refractory to vincristine, prednisone, and anthracycline-based treatments [87–94]. Additionally, the majority of adults who achieve a CR will ultimately relapse. Overall, approximately 65% of adults with ALL will require treatment in the refractory or relapsed setting [6]. The experience at MSKCC in over 130 adult patients with first relapse of ALL indicate a median survival of less than eight months, with less than 5% of these patients alive at five years [6].

These data illustrate the extremely grave prognosis for adult patients with relapsed ALL. For patients with relapsed (or primary refractory) ALL, achieving a second CR (or first CR in primary refractory ALL) followed by a successful transplant strategy is the only realistic chance of cure. While the improvement of salvage therapies is an immediate goal in developing treatment protocols for adult patients with relapsed or refractory ALL, the development of more efficacious induction strategies for newly diagnosed patients should be the ultimate goal.

Treatment strategies for relapsed disease can be divided into two general approaches. The first is to repeat the original vincristine, prednisone, and anthracycline-based induction therapy. Obviously, for primary refractory disease, this approach is inappropriate. The second approach is to utilize a cytarabine-based treatment, in particular, a high-dose Ara-C (HDAC) based regimen. High-dose cytarabine has been used alone and in combination with a variety of other agents, including L-asparaginase, doxorubicin, idarubicin, or mitoxantrone. As a single agent, HDAC can induce a CR in approximately 30% of relapsed or refractory patients [105–107].

The addition of L-asparaginase to HDAC does not appear to significantly improve the CR incidence compared to single-agent HDAC [108–110]. In contrast, complete responses in up to 60% of patients can be achieved when HDAC is used in combination with an anthracycline [111–117]. In general, it appears that the probability of achieving a second CR is greater with an HDAC and anthracycline-based regimen than with a standard vincristine/prednisone encore [6,113,114,116,117]. Of the various combinations of Ara-C and an anthracycline, it is difficult to identify a standard specific regimen [112–116]. Nonetheless, regimens that combine high-dose cytarabine with a single high dose of mitoxantrone or idarubicin have promising activity [116,118]. Patients who attain a CR and have a suitable related allogeneic transplant option should undergo transplant in second complete remission. The role of autologous and matched unrelated transplants in relapsed adult ALL, while an

area of active investigation, has not been definitively established, and should be considered investigational.

For patients who achieve a CR but do not have an appropriate transplant option, postremission strategies can include supportive therapy alone or maintenance therapy with or without preceding consolidation. For those patients who can tolerate some chemotherapy, low-dose maintenance-style treatment may be the best option. Unfortunately, second CRs, even when attained, are usually of brief duration, and essentially all relapsed patients who are not successfully transplanted will die of their leukemia.

The role of bone marrow transplantation in adult ALL

There is considerable debate surrounding the timing of allogeneic transplant in ALL. This dose-intensive treatment approach has the ability to eradicate leukemia in a subset of patients with disease refractory to conventional chemotherapy. Unfortunately, the lack of suitable HLA-matched donors and the morbidity and mortality seen with transplantation limits the utility of this approach [35,119,120].

Analysis of patterns of failure reveal a fundamental difference in allogeneic transplant for ALL compared to AML. Patients with AML (in first or second remission) who undergo an allogeneic transplant fail primarily because of treatment-related complications, such as infection and graft-versus-host disease. Failure due to relapsed AML is unusual. In contrast, patients with ALL experience a significantly higher incidence of relapse following allogeneic transplant, with very few long-term disease-free survivors [119].

This difference between the efficiency of allogeneic transplant in AML compared with ALL may be related to the ability of the donor leukocytes to actively eliminate residual leukemic cells. This graft-versus-leukemic effect (GVL) can be inferred from data demonstrating an inverse relationship between graft-versus-host disease and risk of relapse. One such study from the International Bone Marrow Transplant Registry [121] compared identical twin (syngeneic) to HLA-identical (allogeneic) sibling transplants. This study demonstrated a significantly higher relapse rate in the identical-twin transplants for AML and CML, but not for ALL. Since it is presumed that the graft-versus-leukemia effect would be greater in the HLA-identical sibling transplants, compared to the syngeneic transplants, this study implies that GVL is less important in ALL than in CML or AML. More direct evidence comes from studies employing donor T-lymphocyte infusions for relapses posttransplant. In one study, such infusions resulted in complete remission in 73% of CML patients, 29% of AML patients, and 0% of ALL patients [112]. This lack of GVL may explain in part the high relapse rate and relatively low incidence of long-term disease-free survival following allogeneic transplant for adult ALL.

Review of the published data in allogeneic transplant in relapsed and refractory ALL reveals that only a small fraction of adult patients will be cured

by this modality. The Fred Hutchinson Cancer center reported a five-year disease-free survival of 15% for adult patients transplanted in second CR or beyond [119]. Two large studies including one randomized trial have explored the utility of allogeneic transplant in first CR [120,123]. Unfortunately, these studies failed to demonstrate a survival advantage in the transplant arm compared to maintenance chemotherapy, although one of the studies suggested a benefit for patients in high-risk subsets [120].

Collectively, the existing data suggest that for selected high-risk patients, specifically those with Ph+ ALL, allogeneic transplant from a matched HLA donor should be considered in first CR. For all other patients, reserving allogeneic transplant for the salvage setting (relapsed disease, second CR, etc.) is a reasonable option. Clearly, further clarification of prognostic risk groups is required in order to better identify those patients who may benefit from allogeneic BMT in first CR.

Autologous transplant for adult ALL has proven even less effective than allogeneic transplant [124]. The poor results for patients in second CR or beyond have led to this modality being tested in first CR. Unfortunately, both a nonrandomized and randomized trial have shown no benefit in the auto-BMT arm compared with maintenance chemotherapy [125,126]. This modality should therefore be considered experimental and cannot be recommended outside of a clinical trial.

Mature B-cell ALL

Mature B-cell ALL is generally a disease of young men, frequently presenting with abdominal disease that may then disseminate to the bone marrow. It has a unique morphology (L3 by FAB), immunophenotype (mature B-cell), and karotype (t(8;14)). Recently, several highly successfully regimens have been designed specifically for this entity [82,127,128]. While these regimens vary in their exact treatment plan, they have several features in common. These include rapid cycling of drugs, high-dose methotrexate, high-dose cytarabine, intensive CNS prophylaxis, and no maintenance therapy. Though the reported experience is limited, such regimens appear to cure approximately one half of adult patients with mature B-cell ALL.

Future directions

The challenge in adult ALL investigation is to try to achieve the same degree of success as our colleagues in pediatric oncology. At present, the strategies required to address this challenge remain uncertain. The rationale for the development of novel approaches to ALL comes from historical observations, which have established basic biological characteristics of the disease. Adult ALL, like its pediatric counterpart, can be classified according to risk strata on the basis of immunophenotype and cytogenetics. As previously discussed,

multivariate analyses have identified five distinct adverse prognostic features (Table 3). The first four (age, immunophenotype, presence of the Philadelphia chromosome, and white blood cell count) are dependent upon the patient and the biology of his or her disease. The fifth prognostic feature (time to CR) is to a large extent dependent upon the treatment regimen. Therefore, one potential strategy to improve future treatment results is to utilize a treatment regimen that induces a more rapid CR.

A study conducted at Memorial Sloan-Kettering Cancer Center and New York Medical College treated 37 adult patients with newly diagnosed adult ALL with an induction regimen ('ALL-2' protocol) that did not contain vincristine or prednisone [129]. These patients received Ara-C (3 g/m^2/day by three-hour infusion for five days) and high-dose mitoxantrone (80 mg/m^2 as a single dose on day 3), given with G-CSF support. Patients then received two courses of consolidation. The first (consolidation A) combined vincristine and prednisone with escalating-dose methotrexate and L-asparaginase, and the second consolidation (consolidation B) combined etoposide and cytarabine. All patients received CNS prophylaxis and a standard maintenance regimen.

In comparison to historic controls (treated with a standard regimen, as outlined earlier), the cytarabine/high-dose mitoxantrone induction produced a higher incidence of complete remission (84% vs. 67%) and on average produced a CR in fewer days (34 vs. 54). Despite the very high doses used in the induction regimen, there was no increase in treatment-related deaths. This regimen also produced a cytogenetic CR in 100% of Ph+ patients, compared to 45% in the historic controls, suggesting a potential benefit in this difficult-to-cure subset of patients [129]. At present, this regimen is being evaluated in a multicenter phase III trial, where it is being compared to a standard four-drug regimen.

It is possible that the role of bone marrow transplant for selected patients in first CR will continue to be expanded. In a recent study published by the Fred Hutchinson Cancer Research Group, 18 patients with Ph+ ALL received a matched, unrelated transplant in varying stages of their disease [130]. In the five adult patients transplanted in first remission, four are alive and disease free with 9 to 73 months of follow-up. While these numbers are small, this result is encouraging enough to suggest that matched unrelated transplants be considered for selected adults with Ph+ ALL in first CR who are lacking a suitable related donor.

Another interesting area is the development of vaccines against *bcr-abl* oncogene-derived peptides [131–133]. The t(9;22) produces two chimeric fusion proteins, one of 210 kDa (p210, typically found in CML) and another of 190 kDa (p190, typically found in ALL). The p210 has been shown to elicit a cytolytic human immune response against the *bcr-abl* oncogene-derived peptides [132]. The induction of leukemia-specific T cells reactive with bcr-abl breakpoint peptides is presently being developed as a therapeutic strategy for CML [131]. The fusion peptide most frequently produced in ALL is the p190. Presently, investigators are trying to identify unique immunogenic sequences

of this large chimeric protein in anticipation of developing a leukemia-specific vaccine for patients with Ph+ ALL [133]. While such therapies have not been tested in the clinical setting, strategies directed towards specific adverse prognostic features of the disease represent a rationale approach for the development of new therapeutic options in adult ALL.

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14. Advances in the therapy of the myelodysplastic syndromes

Peter A. Kouides and John M. Bennett

The myelodysplastic syndrome (MDS) has been a most challenging disease for hematologists for decades. In terms of diagnosis, it was less than 20 years ago that the French–American–British (FAB) group developed criteria for the diagnosis and the classification of the myelodysplastic syndromes [1]. The FAB classification has provided a common language for hematologists, morphologists, and pathologists in the investigation of this clonal bone marrow disease, since, studies could be compared in part by stratifying patients into the appropriate MDS subgroup. Since its development, the FAB classification has led to countless studies — from kinder, gentler approaches using various vitamins in hopes of inducing differentiation of the MDS clone to 'brute force' approaches of high-dose therapy with allogeneic marrow rescue. This chapter will review therapeutic studies published in the past four years; readers interested in details of earlier studies can consult several references [2,3].

Therapeutic advances, of course, do not occur in a vacuum. There have been several advances in the pathogenesis and prognosis of MDS that we will discuss prior to reviewing therapeutic studies. These studies in the mechanism of disease and its clinical correlates in terms of prognostic features will undoubtedly be the basis for future therapies.

Apoptosis in MDS

A major advance toward understanding the pathogenesis of MDS has been the observation of apoptosis in MDS, particularly by the group of Raza/Preisler et al. [4–6]. These authors have carried out cell kinetic studies from MDS bone marrow biopsies using intravenous infusions of either iododeoxyuridine or bromodeoxyuridine, or both, and estimating the degree of apoptosis by in situ end-labeling of DNA [5,6]. Virtually all marrows studied demonstrated apoptosis as well as rapid cell proliferation. It is unclear at this time whether apoptosis is this extensive in all cases of MDS [7] and whether it is specific for MDS and no other marrow disease [8]. Nonetheless, the observation of increased apoptosis nicely explains the seemingly opposing findings of marrow hypercellularity but peripheral blood pancytopenia in MDS patients [9]. Intu-

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itively, these findings would appear to be related to levels of apoptosis-related oncogene products such as *c-myc*, which enhances apoptosis, and *bcl-2*, which diminishes apoptosis, as suggested by a study by Rajapksa et al. [10]. Interestingly in that study, patients treated with erythropoietin and granulocyte colony stimulating factor (G-CSF) had reduced apoptosis within CD34+ marrow cells [10]. Other cytokines that may play a role in apoptosis in MDS include tumor necrosis factor alpha, transforming growth factor beta, and interleukin-1 beta converting enzyme, which are all elevated in MDS patients [11]. These cytokines may exert a dual effect of stimulating proliferation of the early CD34+ MDS progenitors while inducing apoptosis in their progeny [6,11]. Certainly, future therapies are being designed to counter this situation, particularly in terms of blocking the common lipid signaling pathway of phosphatidic acid → diacylglycerol that these three cytokines share [12]. Agents that block this pathway include pentoxifylline, lisophylline, and ciprofloxacin [6,12]. Unfortunately, a recent phase II study of ciprofloxacin and pentoxifylline in 14 advanced MDS patients with cytopenias with/without transfusion requirements showed no efficacy [13].

The international scoring system for the prognosis of MDS

Since the advent of the FAB classification of MDS, numerous clinical, pathologic, and biologic factors have been studied in the hope of best predicting the overall survival and acute leukemic transformation risk in MDS patients [14,15]. These factors include the degree of cytopenia, the percent of bone marrow (BM) blasts, BM cytogenetics, serum lactate dehydrogenase level, BM histology for abnormal localization of immature precursors, the marrow immunophenotype, and in vitro bone marrow culture characteristics [14,15]. None of these factors, individually or in combination, has clearly led to a prognostic index with adequate precision. This situation led to the development of the International Prognostic Scoring System (IPSS) for MDS, wherein combined data from seven previously reported studies were analyzed [16]. It was determined that patients could be separated into distinctive subgroups for both median survival and the number of years for a quarter of the respective subgroup to undergo acute leukemic transformation:

Risk subgroup	*Score*	*Median survival (years)*	*Acute myelogenous leukemia (AML) risk*
Low	0	5.7	9.4
Intermediate-1	0.5–1.0	3.5	3.3
Intermediate-2	1.5–2.0	1.2	1.1
High	≥2.5	0.4	0.2

The score is based on the following parameters:

Prognostic variable	*0*	*0.5*	*1.0*	*1.5*	*2.0*
BM blasts(%)	<5	5–10	—	11–20	21–30
Karyotype	Good (normal or 5q- or 20q- or -Y)	Intermediate	Poor (≥3 bnormalities or monosomy 7)		
Cytopenias (Hemoglobin <10 gm%, absolute neutrophil count (ANC) <1500/μL, platelet count <100,000/μL)	0/1	2/3			

Stratification by age less than or greater than 60 separates patients further within the low-risk and intermediate-1 subgroups. The above scoring system was able to discriminate between the subgroups of other categorization systems. This system will undoubtedly be of value in assessing future clinical outcomes and therapies in MDS patients by allowing for better comparisons of therapies by stratifying patients based on the IPSS risk and by comparing the subgroups within a study.

Therapeutic advances

Treatment of MDS can be categorized conceptually into three main approaches, as diagrammed in Figure 1. These approaches include

1. *differentiation of the abnormal clone* by differentiation-induction agents, possibly including cytokines;
2. *suppression of the abnormal clone* by low-dose or standard-dose chemotherapy or perhaps by cytokine therapy via stimulation of residual normal hematopoiesis with subsequent suppression of the malignant hematopoiesis or by activation of the residual normal host immune system by cytokines/cytokine-activated lymphocytes ('immunotherapy'); and
3. *ablation of the abnormal clone* by more intensive therapy via an allogeneic bone marrow transplant (BMT).

The appropriate approach, if any, should depend primarily on three clinical and biological factors:

- severity of the patient's cytopenia
- the patient's age
- the degree of leukemic progression (% marrow blasts)

These factors in turn are reflected in the IPSS [16] described above. The main aim in the elderly patient should be to ameliorate the cytopenias when they

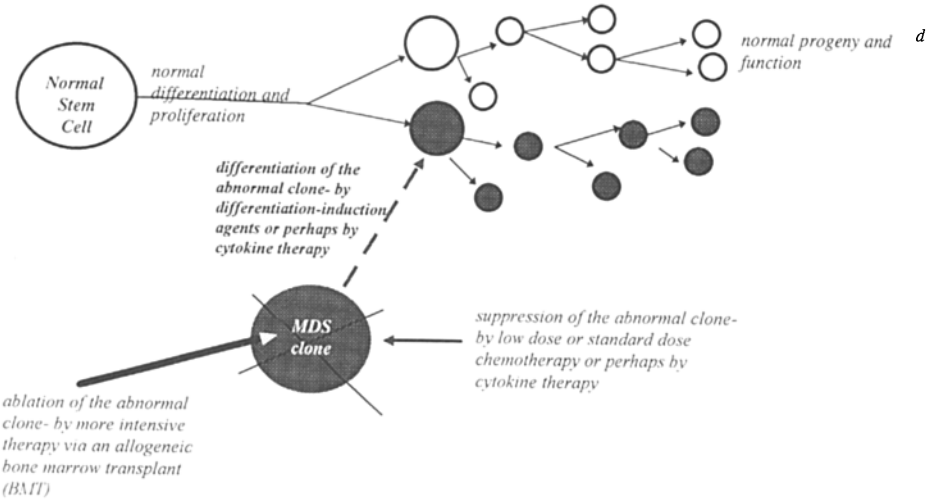

Figure 1. Schema of the various therapeutic approaches in MDS.

become transfusion dependent. However, in the younger patient, the aim may be curative therapy via an allogeneic BMT.

Noncytotoxic/'differentiation' therapy

Studies in the past four years of agents known to induce differentiation in vitro are outlined in Table 1. What these agents have in common is that they are generally noncytotoxic. Since it is not clear that all these agents induce differentiation, we have put this term in quotation marks. Understandably, differentiation historically has been an intensive area of research in MDS, since the ineffective hematopoiesis of MDS represents an impairment in differentiation and several cell lines can be induced to differentiate in vitro [17–19]. Numerous agents have been administered in the hope of inducing differentiation and thereby uncoupling proliferation and differentiation. By and large, these studies have involved patients who by IPSS criteria would probably be in the intermediate-1 and -2 risk groups, i.e., cytopenic and often transfusion dependent, with the FAB subtype typically Refractory Anemia with Excess Blasts (RAEB). The agents used in these studies have included *cis* retinoic acid, hexamethylene bisacetamide, 5 aza 2′-dexoycytidine, vitamin D_3, interferon, and various hemaopoietic growth factors. Initially, there was much enthusiasm with *cis* retinoic acid [20]; however, follow-up studies — including two studies reported in 1995 of a combined 80 patients [21,22] — have not shown it to be effective in improving cytopenias and transfusion dependence. There may be synergism, however, with other differentiating agents and/or cytotoxic agents when these are administered together with *cis* retinoic acid, as suggested by one such promising study [23].

Table 1. Compilation of studies of noncytotoxic/'differentitating' agents in the past four years

Lead author [ref]/year	Agent	Schedule/duration	Complete remission/partial remission	Comments
All-trans retinoic acid studies				
Aul [26]/1993	All-*trans* retinoic acid	• 30 mg/m²/day escalating to 80 mg/m² by week 8 • 3-month trial	• CR: 0/14 • PR: 0/14	5/15 patients actually progressed during the study to AML or worsening MDS
Kurzock [24]/ 1993	All-*trans* retinoic acid	• Dose levels of 10–250 mg/m²/day • 6-week trial	• CR: 0/29 • PR: 1/29	Maximally tolerated dose of 150 mg/m² established, but not an effective dose!
Ohno [25]/1993	All-*trans* retinoic acid	• 45 mg/m² qd • for at least 4 weeks	• CR: 0/23 • PR: 3/23	Authors speculate whether higher doses and longer duration would improve response rate
Visani [27]/1995	All-*trans* retinoic acid	• 45 mg/m² qd • 6-week trial	• 3/10 had rise in Hb >1gm/dL • 5/10 with rise in WBC >500/μL	In all cases, the response was transient peaking at weeks 2–3 therapy — ? exhaustion of ATRA-responding MDS clone or development of resistance
Cambier [28]/ 1996	All-*trans* retinoic acid	• 45 mg/m² qd • 8-week trial	• 4/10 'minor' response	Two of the first four patients had a rapid rise in WBC, as seen in the retinoic acid syndrome in APL patients; 1/2 died from this; the report of retinoic acid syndrome ais of interest; unlike APL, however, there was no evidence for differentiation in in vitro studies
Ganser [30]/1994	All-*trans* retinoic acid + G-CSF	• ATRA ×12 wks with G-CSF added after week 4	• ANC increased in all patients, platelet count in 3/14; only 2/15 had response in terms of anemia	That 5/14 patients had a bilineage response is of interest, but its clinical significance is unclear

Table 1 (continued)

Lead author [ref]/year	Agent	Schedule/duration	Complete remission/partial remission	Comments
Cis-retinoic acid studies				
Letendre [21]/ Mayo Clinic/1995	13-*cis* retinoic acid	• Crossover study with danazol 800 mg po qd × 3 mos. vs. 13-*cis* retinoic acid (CRA) 80 mg po qd × 6 mos. or 200 mg po qd × 3 mos.	• CR: 0/23 • Minor response (less than a PR): 2/22 with low-dose CRA; 1/20 with high-dose CRA • PR: 0/22 with low-dose CRA; 1/20 with higher dose CRA; PR: 1/34 with Danazol	Criteria for response in this study are more strigent than in other studies, with CRA that historically showed a higher response rate
Bourantas [22]/ Greece/1995	13-*cis* retinoic acid	• 10–60 mg/^{2} qd administered in combination vitamin E to reduce side effects • 3 mos. to 5 years	• CR: 0/34 • PR: 4/34	Not very promising
Ferrero [23]/ Italy/1995	13-*cis* retinoic acid + vitamin D_3 + 6TG	• *Cis* retinoic acid 20–40 mg po qd + vitamin D_3 (1–1.5 mcg qd) × 10 weeks with 6TG wks 1–3, 6–8, if BM blast ≥5%	• Response rate 52% (1 complete, 8 partial, 4 minor responses) if <5% blasts • Response rate 61% (12 partial, 6 minor responses) if ≥5% blasts	Combined differentiating therapy seems more effective than previously reported single-agent treatments
Aza/deoxy-cytidine studies				
Silverman [37]/1993	Low-dose 5-azacytidine	75 mg/m^2 IVC × 7 days q 4 wks	Overall response rate (RR) 49% (21/43); CR, 5/43 (12%)	Higher trilineage response (>50% improvement in one third of patients is higher than what is noted with HGFs alone

Wijermans [38]/1997	Low-dose 5-azacytidine	50–75 mg/m² IVC × 3 d	Overall RR 54% (15/29); CR, 8/29 (28%)	Not a differentiating effect; 5 toxic deaths; one infectious/one bleeding
Zagonel [39]/1993	5-aza-2-deoxy-cytidine (DACITABINE)	IVC × 72h or IVSS × 3	4/10 with complete hematological response	Well tolerated: the lack of aplasia with only transient hypoplasia in half suggests differentiation effect
Biologic response modifiers				
Holcombe [20]/1993	Alpha-2a interferon	• 1 million units sc 3 × wk × 16 weeks	• PR = 3/7	—
Stone [121]/1993	Gamma interferon	• 2–20 MU/m²/d × 14 days IVC	• PR/CR = 0/2 MDS patients	Too toxic; hepatotoxicty, renal failure and pulmonary deterioration
Petti [35]/1996	Lymphoblastoid alpha interferon	• 0.5–3.0 MU sc 3 × week × 3 mos.	• PR = 4/17; 2 of these 4 achieved CR after the INF was continued beyond 3 mos.; 2 patients who did not respond after 3 mos. achieved PR after discontinuing the INF	This study is a reminder that therapy with biologic response modifiers do not 'work overnight' if they work at all
Rosenfeld [123]/1997	Roquinimex (linomide), a stimulator of cytotoxic T cells and NK cells	• Orally twice weekly for 12 weeks	• 4/17 with partial improvement in WBC • 2/17 with partial improvement in platelet count	There was an increase in CD8+ and CD3–CD56+ NK cells, but NK and LAK cell activity was not increased; it is possible that a response was not seen because a fair number of these patients had RAEB; Ogata [125] showed that RAEB, unlike RA, patients have educed LAK cell cytoactivity by begin with

The efficacy of all-*trans* retinoic acid (ATRA) in acute promyelocytic leukemia has led to numerous attempts in the past few years to replicate this success with MDS patients. Approximately 100 MDS patients administered ATRA have been reported. In no case was a complete remission achieved [24–28]. Only approximately 10% of the patients had a partial improvement in their peripheral blood counts, and this improvement usually was transient, implying either exhaustion of the ATRA-responsive clone or development of resistance [27]. At least these studies did establish a maximally 'tolerable' dose of 150 mg/m^2/day [24]. The majority of the patients did experience some degree of dermatological toxicity — typically a mild dermatitis and often hypertriglyceridemia. In a study of ATRA in CMML patients, two patients had a rapid rise in white blood cell count with fever and pulmonary signs resembling the retinoic acid syndrome that can be seen in APL patients receiving ATRA [28]. Further cases of retinoic acid syndrome were averted by adding hydroxyurea at the onset of ATRA therapy if the WBC count initially was increased or if it increased on ATRA. There is an intriguing report of a cytopenic MDS patient with trisomy 8 who had a partial response to ATRA with disappearance of the trisomy 8. What is notable is that ATRA was administered to this patient because she had a relative maturation arrest at the promyelocyte stage [29].

At present, a variation on the theme of using ATRA in MDS has been to combine it with other agents such as G-CSF, since at least in AML cells this combination can induce differentiation. Preliminary results by Ganser et al. [30] of using ATRA in combination with G-CSF did not demonstrate a trilineage response. However, these authors did note trilineage responses in a later study of 17 patients who additionally received erythropoietin and alpha-tocopherol, with the number of patients who had increased hemoglobin slightly higher than the proportion seen historically receiving erythropoietin alone [31]. Interestingly, the peripheral blood monocytes of these patients had an improvement in their capacity to secrete cytokines (interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor α [32]. Whether this outcome will translate into reduced risk of infection is unknown.

No further studies of hexamethylene bisacetamide have been reported in the past four years after a number of reports, particularly from Johns Hopkins and Sloan-Kettering, demonstrated some degree of efficacy [33,34]. Interferon continues to be studied. A new twist has been the use of lymphoblastoid interferon alpha, which is a mixture of several types of interferons [35]. Although the sample size of 17 patients was small, 4 of 6 patients who responded partially after three months of treatment went on to a complete remission with continued interferon, while two nonresponders had a partial response after the interferon was discontinued [35]. As for vitamin D_3, we must await results of ongoing clinical studies of the various vitamin D_3 analogues which in vitro show greater antiproliferative and differentiating, effects than 1,25 vitamin$(OH)_2D_3$, with less risk of causing hypercalcemia [18]. There is, how-

ever, a recent case report of low-dose Vitamin D_3, akin to the dose given for osteoporosis, that led to a partial response [36].

Finally, in the past four years, there have continued to be studies of azacytadine and its analogue 5 aza 2′-dexoycytidine in MDS patients, with the rationale that this modest inducer of differentiation in vitro leads to hypomethylation of DNA, which in turn results in transcription of previously quiescent genes that may induce differentiation [37–39]. In patients who respond, an absence of marrow aplasia precedes improvement of the cytopenia, implying a differentiating rather than a cytotoxic effect in vivo [39]. The response rate has been variable, but complete remissions (CRs) have been achieved in 12%–40% [37–39] of patients. Therefore, this agent is a candidate for combination therapy, especially with HGFs — particularly given in vitro studies that demonstrate the ability of azacytidine to restore cytokine responsiveness in leukemic cells [40].

Ongoing studies not yet published in final form may also yield promising results. Agents under investigation that are touted to be antiapoptotic and to induce differentiation include amifostine [41], tamoxifen [42], and recombinant human hemoglobin [43], as well a combination of agents known to affect the phosphatidic lipid signaling pathway (pentoxifylline, lisofylline, ciprofloxacin, and dexamethasone) [12].

Low-dose chemotherapy

Table 2 outlines studies of low-dose chemotherapy in MDS published in the past four years. Historically, it was initially felt that the efficacy of low-dose chemotherapy, particularly ARA-C, was due to a differentiative effect [19], but further studies have demonstrated a predominantly cytotoxic effect. Given the baseline of a total response rate with low-dose ARA-C (LDAC) in up to a third of patients (approximately 16% CR and 21% PR, as reviewed by Cheson [44]), recent studies have attempted to improve upon this outcome by adding additional cytotoxic agents at low doses, such as etoposide [45], 6-TG [46], G-CSF [47], or interleukin-3 [48]. In all cases, the response rate is higher than that seen with LDAC alone but typically not significantly greater [46,48], and usually the response is of a short duration [45]. However, one study of LDAC alone deserves particular attention, since all five patients in the study achieved a complete hematological response. These patients all had deletion of 5q- and were red cell transfusion dependent [49]. Notably, the dysplasia still persisted in the follow-up marrows, as did usually the 5q- abnormality. The authors of this study, as well as ourselves, have no logical explanation for why there could be an ARA-C-specific response in patients with 5q-.

In addition to studies of low-dose ARA-C with or without additional cytotoxic agents or cytokines, several other studies have investigated low-dose chemotherapy using idarubicin [50] (no efficacy), etoposide [51–53] (variable efficacy, with one large randomized study in CMML showing inferiority

Table 2. Compilation of studies of low-dose chemotherapy in the past four years

Lead author [ref]/ year	Agent	Schedule/duration	Complete remission/ partial remission	Comments
Greenberg [50]/1993	Low-dose oral idarubicin	21 days q 5 wks 6 courses	Only 1 PR/42	Despite low dose, only 8/42 patients received the full 6 courses because of myelosuppression; ? higher dose needed
Ogata [52]/1993	Low-dose etoposide	50 mg q2–7 × week	4/6 patients (all RAEB-T) were partial responders	Small sample size limits conclusions; further study is warranted
Omoto [54]/1996	Low-dose melphalan	Daily until bone marrow blast progression	Overall response rate of 57%; CR 7/21 (33%)	Majority of the responders had marrow hypoplasia pretherapy; no marrow suppression or progression of pancytopenia noted, suggesting that melphalan has the ability to induce differentiation or kill leukemic cells selectively
Juneja [49]/1994	Low-dose ARA-C; all 5 patients had at least the cytogenetic abnormality 5q-	7.5–20 mg/m^2 bid or bi-weekly for 10–39 days	9 CRs and 1 PR induced in 5 patients	There is seemingly a specificity of response to low-dose ARA-C based on the underlying cytogenetic abnormality cytogenetic abnormality

Kuriya [45]/1996	Low-dose ARA-C and etoposide	For 9–21d; previously higher doses used in 2°AML	Overall response 8/10; 6/10 CR! but short duration < 12 mos	Effect probably via cytotoxicity since all patients achieved hypoplasia; unfortunately, the agents were given IV
Munshi [46]/1997	Low-dose ARA-C and low-dose 6-thioguanine	100 mg qwk + 6TG 80 mg po d2–5	Peripheral blast count reduced to mean of 5% (0–23%); platelet count 24,000/μL pre-therapy to 95,000/μL posttherapy; 10/12 RBC transfusion independent	Mild toxicity–initial cytopenias, 1/12 neutropenic fever. Conclusion: remarkably well-tolerated effective palliative therapy for high-risk MDS or AML; note that it does not work overnight — therapy needed for 6–10 wks before response
Wattel [53]/1996	Hydroxyurea, etoposide	1 g hydrea vs. VP-16 50 mg poTIW	n = 105; overall response rate 60% with hydrea vs. 36% with VP-16 with median survival time (MST) 20 mos. (Hydrea) vs. 9 mos. (VP-16)	Somewhat surprising results given encouraging previous pilot studies (228,232) of VP-16 in CMML
Gerhartz [48]/1996	Low-dose ARA-C + IL-3	3 cycles of ARA-C bid d 1-14 + IL-3 d 8–21	Overall response rate of 42%; CR 5/31 (16%)	This novel regimen was well-tolerated; further study is warranted
Im [47]/1994	Low-dose ARA-C + G-CSF	Low-dose ARA-C (20 mg/m^2 q12 hrs × 5 days) + G-CSF (125 mcg SQ d7–17)	Overall response rate of 62%; CR 4/13 (31%)	Given typical overall response rate of 37%, the higher response rate may suggest a synergistic/additive effect of ARA-C + G-CSF

compared to hydroxyurea [53]), and melphalan [54]. Akin to the 5q- response to ARA-C, marrow hypocellularity appears to correlate in the laboratory with a response to low-dose melphalan chemotherapy. Surprisingly, despite preexisting marrow hypoplasia, responders did not have prolonged pancytopenia, suggesting a differentiative or selective antileukemic cell effect rather than cytotoxicity in general as the mechanism of response [54]. Since hypocellular MDS makes up only 10%–20% of MDS cases [55], however, the impact of melphalan in MDS may not be substantial, and further studies are clearly needed.

Intensive chemotherapy (Table 3)

Recent studies employing intensive AML-type induction regimens continue to be associated with a lower CR rate and shorter remission duration than in de novo AML [56–58], although some studies have shown comparable CR rates and remission duration [59,60]. Since one major negative factor in intensive chemotherapy for MDS, namely, age, cannot be manipulated, investigators have instead tried to manipulate 1) the longer duration of aplasia/hypoplasia, by administering G-CSF [56] or GM-CSF [57], and 2) multidrug resistance, by adding agents such as etoposide [56] and administering interleukin-2 post-CR to lengthen the duration of CR by hopefully stimulating immune surveillance [56].

In addition to intensive AML-type regimens, other investigators have tried novel agents not previously administered in MDS, such as 2-chloro-deoxyadenosine [61] (data are too preliminary), homoharringtonine [62] (mild efficacy), and topotecan [63]. Topotecan, a topoisomerase I inhibitor, is the most promising of these, with a 28% CR rate in 47 patients (n = 22 RAEB/RAEB-T, n = 25 CMML) — one of the highest CR rates for single-agent therapy in MDS. Yet enthusiasm is tempered by the fact that 19% of the patients died during the induction period, six of these with marrow hypoplasia and three with residual disease. Whether this toxic rate will be abrogated by a lower-dose oral form of topotecan or lower doses in combination with other agents but without compromising efficacy is an important question that must be answered prior to making topotecan the chemotherapy agent of choice in high-risk MDS patients (i.e., those requiring treatment).

Allogeneic bone marrow transplantation

The high treatment-related mortality (TRM) of 30%–50% associated with allogeneic bone marrow transplantation in MDS is the price paid for the possibility of cure with 20%–70% event-free survival (see Table 4 for a compilation of studies) [58]. This wide range is dependent on the exact age of the patient, the FAB subtype (RA/RARS is best [64]), the duration of the disease (shorter is preferable [65]), the percent marrow blasts (<5% blasts is best [66]), and the presence and degree of cytogenetic abnormalities (none, of

course, is best [65], though some studies have shown that one abnormality is best! [66]) [58].

In these patients, who are usually between the ages of 45 and 55, many questions remain even after the sibling is determined to be histocompatible

1. What should the upper age limit be for allogeneic BMT? The question of upper age limit is most difficult to answer, since the median age of MDS patients transplanted to date has been less than 40 years old. Consequently, one could argue that even the 'best' results are not very applicable for most MDS patients. However, data are accruing, though mostly from autologous bone marrow transplantation studies, showing that age (greater than 40 but less than 60 years) is not a major poor prognostic factor [67].

2. Should the conditioning regimen be a nonradiation regimen so as to decrease the incidence of TRM? It now appears that a nonradiation regimen is at least equivalent to the historical standard of a radiation-based regimen [65,68,69]. However, the degree to which it reduces TRM without increasing the risk of relapse is still unclear at this time.

3. Should the bone marrow be T-cell depleted so as to decrease the incidence of graft-versus-host disease, particularly in the 'older' BMT candidate? The question of T-cell depletion has not been extensively studied [70]. On the other hand, there are several reports of successful donor buffy-coat infusions for relapse after allogeneic BMT for MDS [71–73]. This graft-versus-leukemia effect may be exploited with peripheral-blood allogeneic BMT, as opposed to marrow-derived BMT [74].

4. Should the patient receive intensive chemotherapy initially in the hope of reducing the leukemic burden and subsequent risk of relapse post-BMT? The risk of initial intensive chemotherapy versus the risk of relapse post-BMT appears to be a trade-off, since shorter disease duration, i.e., faster time to transplant, is a favorable prognostic factor [65]. Since RAEB/RAEB-T patients with a normal karyotype have an apparently equivalent rate of successful remission induction therapy to de novo AML patients [75–77], it may be reasonable to proceed with induction-remission therapy first in these patients prior to allogeneic BMT in the hope of ultimately reducing the risk of post-BMT relapse as much as possible. Along these lines, Estey et al., in their cohort of high-risk MDS patients (RAEB $n = 20$, RAEB-T $n = 61$, and CMML $n = 3$) treated at their institution with AML-type therapy, noted a profound difference in the CR rate and disease-free survival (DFS) at three years based on the karyotype. A favorable karyotype (diploid or t(8;21) or inversion 16; $n = 48$) resulted in CR of 79% and DFS of 65%, while an unfavorable karyotype resulted in CR ($n = 102$) of 60% and DFS of just 5% [77].

Table 3. Compilation of standard/intensive-dose chemotherapy in the past four years

Lead author [ref]/ year	Agent	Schedule/duration	Complete remission partial remission	Comments
Beran [63]/1996	Topetecan	IVC	CR: 6/22 (27%) MDS; 7/25 (28%) CMML	Interestingly, patients with cytogenetic abnormalities achieving CR had clearance of the cytogenetic abnormalities; relatively toxic, though, as 9 patients died (19%) died during induction, but as such we agree with the authors that it has an acceptable toxicity profile; MST equivalent to what RAEBs see anyway; *nonetheless*, few other agents have shown this degree of single-agent activity; future studies in combination with hypomethylating agents maybe of interest given in vitro synergy
Feldman [62]/ 1996	Homo-harringtonine	IVC × 9 days	CR 3/15	Cytotoxic effect, as all CRs first entered aplastic phase prior to CR — even the authors, however, do not advise further evaluation of HHT in MDS
AML-type induction regimens				
Ganser [56]/1993	Idarubicin + cytarabine + VP-16	In 3 + 7 + 3 →7 fashion fashion followed by G-CSF then postremission IL-2	n = 19, CR rate 47%, with only one toxic death	Low toxic death rate is almost too good to be true; the use of interleukin-2 for maintaining the postremission duration is of interest

Bernstein [59]/ 1996	Adriamycin/ mitoxantrone + cytarabine	A 30–45 mg/m²/d (M 12 mg/m²/d) + C 100–200 mg/m²/d in 3 + 7 fashion	n = 33, CR 79% similar to 68% in the AML patients (n = 874), with CR duration 11 mos	Not a true prospective study, so it has considerable shortcomings in that it is a retrospective study of patients entered into a national AML trial registered as de novo AML then on re-review discovered to have MDS
Econo-moupoulos [57]/1996	Idarubicin + ARA-C	Idarubicin 6 mg/m² + ARA-C 200 mg/m² bid × 5d then GM-CSF qd d 6+with post-CR × 2 cycles	Overall response rate 16/22 (73%); CR 12/22 (55%); 4 PR with 12 mos. median duration of CR	These results are comparable to other studies of RAEB and RAEB-T with aggressive AML-type regimens
Ruutu [60]/1997	Prospective multicenter trial of cytarabine + idarubicin	Cytarabine (100 mg/m²) q12 × 7 days plus idarubicin 12 mg/m² on days 5–7; patients in remission received 2 cycles of cytarabine cays 1–5 plus idarubicin on days 5	CR 11/19 (58%); Follow-up: 13–48 mos. (median 33 mos.) with 7/40 surviving, of which 4 were MDS patients, all who underwent allo BMT; median survival of chemotherapy-treated patients only was 12 mos.	Good news, relatively high CR rate; bad news, short survival — i.e., need better postremission strategies

Table 4. Compilation of BMT studies in the past four years

Study	BMT type	Conditioning regimen	*n*/age/sample	DFS/overall	Comments
Autologous bone marrow transplantation					
LaPorte [81]/1993	Autologous bone marrow with mafosfamide purging	Mostly Cy + TBI	$n = 7$; 44 yrs old; they had 2° AMLs in CR; i.e., this was a select group of patients, as they had to be in CR prior to BMT	1 died periBMT; 2/7 alive and well @ 10+ and 28 mos.	Despite prolonged thrombocytopenia with median recovery at 120 days (and WBC recovery at 41 days!), regeneration of hematopoiesis occurred in all patients
Demuynck [82]/1996	Autologous peripheral blood stem cells	Busulfan and cytoxan	A very select group of patients, as they all have had to achieve CR prior to mobilization	No long-term survival results available; only 5 patients transplanted — ANC recovery to 500 just 14 days; platelet recovery 41 days; one case of marrow failure	The one case of marrow failure is a concern; nonetheless, the faster WBC and platelet count recovery than with automarrow rescue makes a case for peripheral rather than autologous stem cells as the source of rescue in these patients
Allogeneic bone marrow transplantation					
Ratanathar-athorn [68]/ 1993	Mostly haploidentical	Busulfan ± ARA-C/ cytoxan	$n = 27$; 33 yrs; short time between dx of MDS time to BMT (5.6 mos.); half the patients were RAEBs, others were RA with transfusion dependence	56% ± 21%; 10/21 TRM; no significance in terms of FAB type; too short f/u for assessing for relapse	Very low relapse rate with busulfan-based regimen is of interest

O'Donnell [69]/1995	All haploidentical	Bu-Cy	n = 38; 35 yrs (5–55); relatively advanced-RAEB-Ts	37% DFS @ 2 yrs; O.S. at 2 yrs was 45% (CI 0.3–0.61) with 24% relapse rate; 19/38 TRM; no significance in terms of FAB type	Relapse rate is similar to initial Seattle report of radiation based conditioning; neither marrow blasts nor age (but close as p = 0.07) nor cytogenetics a poor prognostic factors
Anderson [65]/1996	All unrelated; 34/52 haploidentical	n = 33-TBI; n = 19 bucy	n = 52; 19 with 2 AML; 17 with >5% BM blasts; 33 yrs median age	2-yr actuarial survival/ DFS/relapse/TRM 40%/38%/28%/48%; ↑age, disease duration, CMV negativity and low neutrophil count were four predictors of TRM	Too good to be true in that results are similar to what is seen with related sibs; also too good to believe that of the 19 survivors, 16 have performance status of 90%–100%
Sutton [66]/ 1996	All haploidentical	Majority TBI-based	n = 71; 16 RA, 27 RAEB, 28 RAEB-T	7-yr EFS 32%, relapse rate 38%, and TRM 39%	The high rate of relapse noted in he RAEB & RAEB-T subgroups have compelled subgroups have compelled these investigators to now study in randomized fashion the role of intersive chemotherapy prior to BMT

5. Is unrelated bone marrow transplantation a viable option in this age group? Three studies, all reported in 1996, appear to have results equivalent to those of sibling matched BMT [78–80], but the total number of such patients is relatively small. Intuitively, it is hard to believe that results would be comparable, although there may be a trade-off of reduced relapse but higher TRM.

Conclusion. Finally, there are now published reports (12 patients total) of autologous stem cell transplantation in the therapy of MDS [81,82]. However, follow-up is relatively short, thereby precluding conclusions regarding the place of this therapy in the field of bone marrow transplantation for MDS. Importantly, and counterintuitively, there probably has been long-term repopulation by marrow rescue after myeloablation, suggesting that normal stem cells/progenitors must be present; indeed, polyclonal hematopoiesis has been demonstrated in peripheral blood stem cell harvests [83]. Similar to the situation in chronic-phase CML, it may be appropriate to consider peripheral blood stem cell mobilization relatively early in the course of this disease on the assumption that there will be a greater proportion of nonmalignant progenitors.

Hematopoietic growth factors

As recently reported by Ganser and Heolzer [84] in a total of 78 patients receiving G-CSF and 263 receiving GM-CSF, a sufficient rise in the neutrophil count was noted in 76% of the patients treated with GM-CSF (two thirds of patients respond to very low doses of GM-CSF [85]) and in 90% of the patients with G-CSF. However, there still have been no direct comparative studies to date. Most important, there have been no fully published randomized, placebo-controlled, double-blind studies of GM- or G-CSF versus supportive care, though one small randomized study of GM-CSF has been reported in abstract form [86] to have decreased the infection rate, and a multicenter study of G-CSF ($n = 102$), also presented only in abstract form, showed no difference in the progression rate to acute leukemia [87]. However, the G-CSF-treated RAEB patients had decreased survival of 10.4 months compared to the observation-arm survival of 21.4 months ($p = 0.02$). The survival of 10 months for the G-CSF arm was actually equivalent to historical controls of the survival of RAEB. In this study, the difference between these arms was attributed to an imbalance of high-risk RAEB patients by the Sanz prognostic system (i.e., 15% more high-risk RAEB patients were randomized to G-CSF than to supportive care).

On occasion, trilineage responses have been noted with G-CSF [88,89] and GM-CSF [90], and in vitro, there can be synergism of HGFs on normal and MDS progenitors [84]. These two observations have prompted studies of either of these cytokines with erythropoietin in the hope of improving the baseline 15%–25% response rate [84,91] seen with erythropoietin alone. By

and large, pretreatment with six weeks of G-CSF [92,93] or GM-CSF [94–96] followed by erythropoietin will lead to a 1.5- to 2.0-fold increase in the response rate to erythropoietin in terms of increasing the hemoglobin by greater than 2.0 gm% and/or reducing the red cell transfusion requirements [92–96]. This approach appears worth trying in the moderately to severely anemic MDS patient. However, studies have shown that such patients have approximately a 50% or higher probability of responding to erythropoietin alone if they have the following clinical characteristics: FAB subtype refractory anemia [97–99], baseline serum erythropoietin level less than 100 IU/L [97,100–102], red cell transfusion independence [91], and early response (i.e., within the first eight weeks) [102]. In a meta-analysis by Hellstrom-Lindberg of 205 patients, no patients with RARS and a serum erythropoietin level of more than 200 IU/L responded [91]. However, other investigators have not reported such a profound lack of response in RARS patients [99,103]. It is hoped that as our understanding of the molecular defects in erythropoiesis in MDS is elucidated [104,105], therapies will be developed that can correct/ameliorate these defects. One promising area is the study of the erythropoietin receptor signal transduction pathway, which appears to be significantly impaired in MDS [106].

Regarding interleukin-3 (IL-3), earlier studies have reported its ability to induce trilineage in a small proportion of pancytopenic MDS patients [107,108]. A logical extension of such studies has been to combine IL-3 with erythropoietin; however, only 4 of 30 patients had an erythroid response [84]. A more promising cytokine that may be quite effective in stimulating erythropoiesis is stem cell factor (SCF), since 1) MDS patients have reduced stem cell factor concentrations [109] and 2) colony formation of MDS progenitors is improved in vitro by the combination of SCF and erythropoietin [110–112].

Finally, interleukin-6 has entered clinical studies of MDS patients. In a phase I study, 8 of 22 patients experienced a transient improvement in the platelet count, but the treatment was relatively toxic [113]. The hope now, of course, is that a much better response rate with less toxicity will be seen once phase I/II studies begin with thrombopoietin for thrombocytopenic MDS patients. By and large, there appears to be an inverse relationship between circulating thrombopoietin levels and the platelet count, akin to erythropoietin levels in anemic MDS patients, so it is not clear that thrombopoietin will be effective [114].

Miscellaneous therapies

Androgens have been tried for years in anemic MDS patients without much success, but nonetheless, studies of androgen use in MDS continue. Danazol continues to be studied, with two recent studies of a combined 110 patients; a partial response was noted in only four of these patients [21,115]. Generally, these patients did not have platelet counts less than 50,000/μL. Based on

previous studies that appeared to show some degree of response in the platelet count in thrombocytopenic patients, a study by Wattel et al. included only patients with platelet counts less than 50,000/μL (as well as less than 10% marrow blasts). These authors administered either danazol or fluoxymestrone, with 11 of 20 patients having at least a 30,000/μL incremental rise in their platelet count; of the six who had bleeding pretherapy, all had resolution of bleeding [116]. In another study by this same group, in which 9 of 26 evaluable patients responded to danazol, there was no correlation of the responders with antiplatelet antibodies or platelet lifespan [117]. An earlier study showed that thrombocytopenic MDS patients responding to danazol had evidence for an autoimmune component [118].

Interestingly, transfusion dependence may be lessened by subcutaneous desferrioxamine [119] — a reminder that, particularly in low-risk MDS patients, iron stores should be assessed and consideration given for iron chelation therapy. This approach may not only reduce the morbidity of iron overload that may in time come into play due to the relatively long overall survival in the IPSS subgroup but also may reduce the transfusion requirement in the majority of these 'de-ironed' patients, as demonstrated by Jensen et al. [119].

Other therapies fall into the category of immunomodulating agents and include, as previously mentioned, interferon [35,120,121], interleukin-2 [122], roquinimex [123] (see Table 1), and cyclosporine [124]. Cyclosporine with antithymocyte globulin has been used in two hypoplastic MDS patients, with clinical improvement (achieving transfusion independence) and disappearance of the marrow dysplasia.

Summary

The study of the proliferation and differentiation of the MDS clone at the molecular level, including the details of apoptosis, may hopefully lead to more effective differentiation–induction/antiapoptotic agents. The study of the cytokines at the cellular/molecular level may lead to more effective trials of combination therapy with differentiation–induction agents, chemotherapy, and/or early-acting cytokines. Further phenotypic characterization of the MDS clone may lead to negative selection of these cells or positive selection of normal stem cells as part of an autotransplant strategy, as is presently being done in chronic-phase chronic myeologenous leukemia. The use of agents such as the topoisomerase I inhibitors (e.g., topotecan), which have mechanisms of action disparate from agents already used in MDS, may increase the efficacy of chemotherapy for MDS. The further clinical refinements in reducing treatment-related mortality and the study of T cells at the molecular level may hopefully lead to improvement in the prevention and therapy of graft-versus-host disease, in turn increasing the upper age limit of allogeneic BMT for MDS and increasing the feasibility of matched unrelated allogeneic BMT.

Is the patient in IPSS high risk or intermediate-2 risk group??

- **Yes → Is the patient < age 50 with a haploidentical sibling??**
  - Yes→ allogeneic BMT and consider attempt of AML-type induction therapy pre-BMT if RAEB/RAEB-T with favorable karyotype (diploid or t(8;21) or inv.16)
  - No→ consider trial of topetecan or AML-type induction therapy
    → if < 40 years old, consider unrelated BMT
- **No→ are they moderately to severely cytopenic?**
  - Yes → → <u>If anemic to point of RBC dependence or Hematocrit <28-30 %</u>:
    - trial of erythropoietin 150 u sc/kg TIW x 3 months if serum erythropoietin level < 100 U/L; if no response then d/c erythropoietin and pre-treat with G- or GM-CSF qd x 6 weeks followed by re-initiation of erythropoietin
    - subcutaneous Desferral if serum ferritin >500 and % iron saturation >60%

    → <u>If thrombocytopenic</u> <50,000/μl platelets
    - consider trial of Danazol 200 mg po tid

    → <u>If neutropenic</u> (<1,000 ANC) <u>with</u> recurrent infections:
    - G- or GM-CSF titrating from 1-7 x week subcutaneously

    → <u>If bone marrow is hypoplastic</u> consider trial of:
    - low dose daily melphalan 2 mg po qd
    - oral cyclosporine ± anti-thymocyte globulin
  - No→" watch and wait"

Figure 2. Summary approach of the treatment of MDS.

At present, we can tailor the approach to a MDS patient based on his or her IPSS risk stratification, degree of cytopenia, and age, as outlined in Figure 2.

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Addendum to Chapter 10: AIDS-associated lymphoma

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Due to the addition of new material to Chapter 10, the last two pages of references have been moved to the back of the volume to allow for all updates.

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