Birkhäuser Advances in Infectious Diseases
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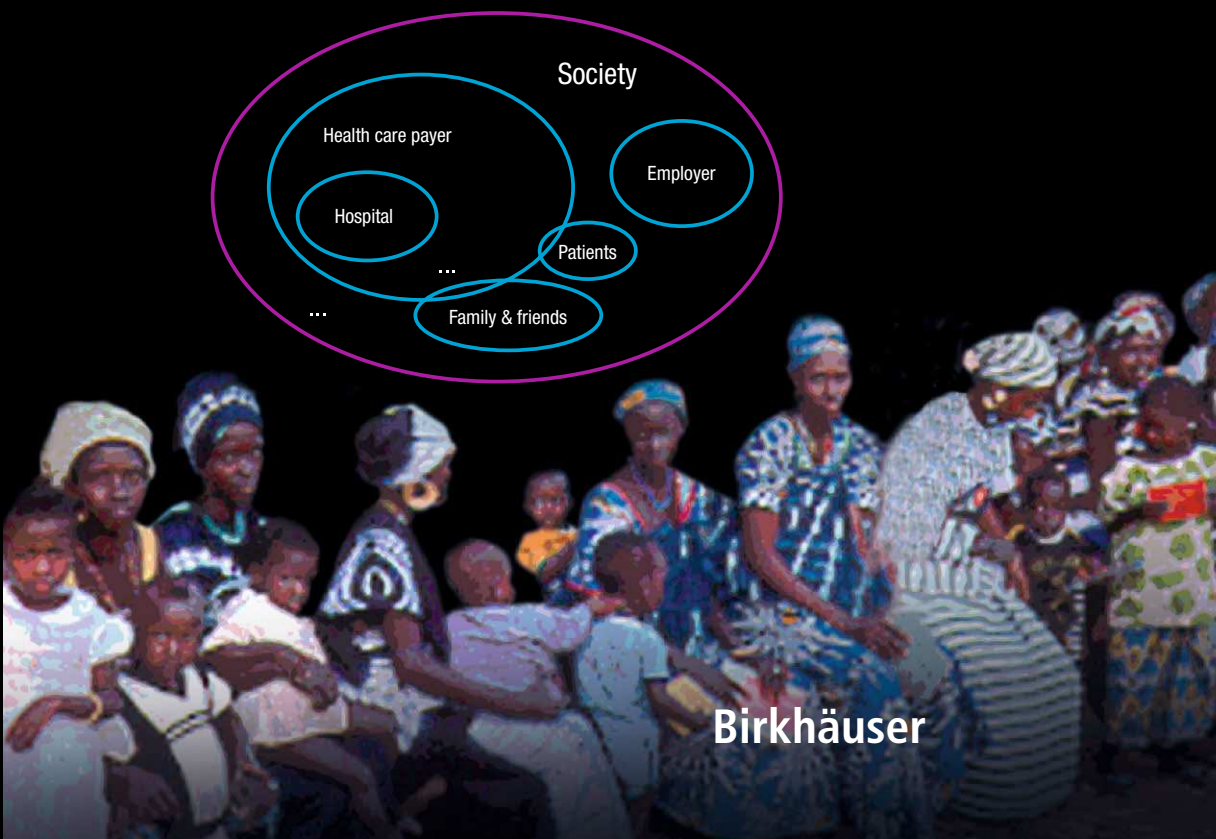
Series Editors

The Grand Challenge for the Future

Vaccines for Poverty-Related Diseases from Bench to Field

Stefan H. E. Kaufmann
Paul-Henri Lambert
Editors



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Edited by S. H. E. Kaufmann and P.-H. Lambert

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Introductory remarks: Towards relevant vaccinology

It is a dream for all scientists engaged in vaccine research to quickly identify a gene product or genetically engineer a microorganism and to find out that this product represents a potential vaccine against a major infectious disease. It is often a nightmare for the same scientist to experience the hurdles that have to be overcome to move this marvellous product towards clinical trials. Most distressful may even be the rising awareness that a fully successful candidate may end up on the list of vaccines waiting for the unlikely introduction into vaccination programs.

The main goal of this book is to review various aspects related to vaccinology and to appreciate how they apply to major infectious diseases associated with poverty. This approach will take us from vaccine research and development efforts for malaria, AIDS and tuberculosis vaccines to issues critical to introduction of these candidates into national vaccination programs.

These reviews show how, within a few decades, vaccinology has emerged as a unique discipline through the increasing confrontation of the scientists who deal with vaccine research with the intricate network of multiple obstacles to be overcome. Issues range from immunology to epidemiology, from genetics to ecology, from economics to industrial engineering, from social sciences to primary health care. Until a few years ago, a vaccine would largely be developed on the basis of its technical feasibility within a decent time frame. Surprisingly little consideration was given at onset to the relative need for the vaccine at public health level or to specific practical issues that could direct its future use. This often led to wrong market analyses. For example, initial marketing studies for a hepatitis B vaccine concluded that use in high-risk groups and medical personnel would be the primary indications. The mere fact that this vaccine was becoming available, however, led public health experts to realize its potential benefit for developing countries where the disease is highly endemic, and to recommend universal vaccination programs.

This development has had remarkable consequences. It is now inconceivable to engage at any level of vaccine R&D for poverty-associated infectious diseases without some knowledge of the broad range of related issues which have to be considered.

Novel vaccination strategies for poverty-related diseases must deal at a global and comprehensive level with the specific issues related to both the target microorganism and the target population.

Antigenic diversity, naturally occurring mutations and microbial selection under immunological pressure do influence the global epidemiology of microbial pathogens targeted by novel vaccination strategies. This is well appreciated for influenza and a major impediment to the design of optimal vaccination strategies for diseases intrinsically associated with antigenic variation, including HIV and malaria. The multiplicity of antigenic variants may open the way to "replacement" strains. For example, new pneumococcal vaccines, which only prevent a limited number of dominant serotypes, can effectively decrease the infant carrier rate for the serotypes covered by the vaccine. However, this may lead to colonization by pneumococci of different serotypes not included in the vaccine formulation. Such replacement phenomena necessitate the continuing monitoring of pneumococcal strains and, if needed, regular changes in the vaccine formulation.

Equally important, the nature of the target population influences the selection of vaccination strategies in conjunction with the definition of epidemiological patterns. For a number of childhood diseases, the period of greatest vulnerability to infection and disease is within the first few years after birth. Vaccine-induced long-term memory is not always critical, since after a basic level of vaccination, re-exposure to the antigens or re-infection can 'boost' the immune response. However, when dealing with poverty-related diseases, one has to face the fact that infants and adults, throughout much of their lifetime, can be at risk for infection. How long can a vaccine given in early life confer protection? Can we expect that infant immunization protects against HIV/AIDS for 20–30 years? How can immunological memory be generated and sustained? Can it be achieved with subunit vaccines, or will immunization with live, attenuated vectors be necessary, with potentially greater risks of adverse effects? Or should we favour combinatory strategies comprising heterologous prime-boost regimes? These are just a few of the questions that do await answers in the near future.

We have learned a great deal about how to deliver vaccines to toddlers and infants to protect them against childhood infectious diseases. Extraordinary logistical challenges had to be solved and ways of integrating immunization programs into public health and primary care programs had to be developed. As one of the results of these efforts, the level of vaccine coverage in developing countries is now far better for vaccines given soon after birth (e.g., BCG, hepatitis B, polio) than for those given later in life. Thus, in addition to protecting against early-occurring infections, any new poverty-related disease vaccine that is found to be efficacious in the first weeks of life will have a remarkable competitive advantage. As discussed in this book, the immunological challenge of engendering potent protective immune responses in newborns is far from negligible.

Vaccination of immunodeficient individuals also poses unique problems. The risk of adverse events needs to be carefully evaluated, particularly if live attenuated vaccines cannot be controlled and cause disease in immunocompromised vaccinees. Because of the long latency of HIV infection before symptoms of AIDS develop, the possibility exists to induce significant protection prior to major immunodeficiency. However, should the level of vaccine-induced immunity fall short of that achieved in the general population, potential implications for transmission and persistence of infection need to be assessed.

Additional challenges reside in the pressing need for rapid assessment of clinical efficacy. Vaccine trials represent a long, complex and expensive endeavour. Surrogates of protection do exist for a number of vaccines currently in use, because they are thought to provide protection by means of neutralizing antibodies which can be easily assessed. However, protection against major poverty-related disease is likely to require multiple and complex mechanisms, including antibodies and cell-mediated immunity. The definition of relevant correlates of protection in these infections remains an area of considerable concern which needs to be addressed in the near future.

In several industrialized countries, public resistance to the addition of new vaccines to the existing paediatric schedule is on the rise. This is often reflected by parental rejection of what is resented as medical "aggression" for their healthy child. It is also directly related to the number of injections required for completing infant vaccination plans. In several European countries, this feeling is reinforced by particular medical groups, who are proponents of new "natural" preventive or therapeutic approaches. These negative reactions partly mirror a decreased perception of the risk of infectious disease in countries where efficient disease control or elimination strategies have been successful.

Although it is likely that such considerations have a lower penetration in developing countries, misperceptions of vaccine safety increasingly contribute to vaccine failures as illustrated by the recent re-emergence of polio in Kano (Nigeria/Niger) following suspension of polio vaccination. Better information is the key to success in this area and this is the topic of an important chapter of this volume.

Finally, numerous challenges are economic in nature. At the industrial level, vaccines increasingly appear as low-profit products, with an increasing litigation risk, as compared to some of the new high-return blockbusting drugs, often produced by another section of the same giant pharmaceutical company. In many developing countries, economic obstacles still limit the use of existing vaccines and no rapid solution can be expected without international aid and significant financing plans. It is hoped that the true value of preventive vaccination will be taken into proper consideration at political and general public levels in both developing and industrialized countries. Greater recognition of the social value of prophylactic vaccina-

tion will be essential in order to let humankind benefit from the considerable scientific and economic efforts that underpin the development of new vaccines against poverty-related diseases.

We are now at a time when relevant vaccinology should prevail. The scientific community is developing an understanding of how to engage the immune response in molecular and cellular terms to provide protection against the target disease. However, the specific needs for the vaccine, the various epidemiological patterns to be faced, the target population foreseen for vaccination and related economic aspects should be equally considered at the time of vaccine design. How will the new and effective vaccine be used? How will target populations be reached? What could be the public acceptance? Will high priority be given to this vaccine in public health policies? These are the realms of knowledge that we see as the "grand challenge" for vaccines and vaccination.

We would like to sincerely thank the staff of Birkhäuser Publishers, and notably Dr. Beatrice Menz, for editing this volume of the "Advances in Infectious Diseases" series, as well as our secretaries Yvonne Bennett, Souraya Sibaei and Gaby Roetzer for their extraordinary help. Most of all we would like to thank all our colleagues who generously shared their knowledge in the broad interdisciplinary field of relevant vaccinology with us. It is only through their in-depth knowledge and their willingness to share their experience with us that we were able to compile this volume on the grand challenge for the future, namely vaccine development for poverty-related diseases from the bench to the field.

Berlin/Geneva, January 2005

Stefan H. E. Kaufmann
Paul-Henry Lambert

Background

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Economic aspects of vaccines and vaccination: a global perspective

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Introduction

A basic principle of economic decision-making is that in an environment of scarce resources choices have to be made in the allocation of these resources. This principle also applies to the provision of health care. The share of health-care expenditures in the Gross Domestic Product (GDP) of most industrialised countries has increased from 3%-5% in the early sixties to 7%–11% in 2001 (from 5% to 14% in the USA) [1] This rise has been attributed to medical advances (increasing the number and technological complexity of medical interventions), population aging, sociological changes (more, but smaller families and less familial support for the elderly) and insufficient productivity increases in the services sector. In less wealthy economies, medical decision-makers are faced with a smaller margin, and such a rise in health-care spending has not been observed yet. Basically, the richer a country, the more it can afford (in nominal and in relative terms) to spend on health care.

The two-way interaction between health and economic development is generally explained as follows. The healthier the population, the more adults can contribute to society by productive activity (i.e., work creating a surplus value in terms of capital gains and human resources), as well as by raising children in a stable environment, thus ensuring continued economic development. The process of economic development itself creates conditions (education, employment, infrastructure (including safe water and sanitation)), which provide a basis for continued improvement in longevity and health-related quality of life [2]. Individual good health can be seen as the product of some unknown complex function to which health care is only one of the inputs. Other important inputs are: life-style variables (eating habits, smoking, etc.), environmental factors (urbanisation, climatic conditions, etc.), income, education and genetic predestination. Furthermore, expenditures on health are not necessarily put to use in the most efficient

way. In this respect a distinction can be made between technical efficiency (providing maximal health care for a given cost, or delivering a certain service at minimal cost) and allocative efficiency (the distribution of resources across alternative services so as to maximise health gains, in accordance with preferences). Finally, though much may be spent on health care, not all people may have equal access to health care of the same quality. Indeed inequities in the consumption of health care may also interfere with the overall allocative efficiency of the system, and create inequities in health *per se*. Therefore greater expenditures on health care are no guarantee for more global health. It should be noted that these observations do not plead for a reduction or containment of health-care budgets, but rather for a way of spending that ensures that societal goals are met. In order to achieve this, welfare economists focus research on two broad topics: efficiency and equity. Efficiency relates to choosing options that maximise utility from marginal expenditures (i.e., by optimising the production process of health, for which health care is one of the inputs). Equity relates to the fair distribution of all aspects related to health across members in society (e.g., equal access to care). Clearly, there may exist a trade-off between efficiency and equity and giving priority to either of them is a normative issue that should be decided by social and political debate.

Vaccination is undoubtedly one of the major contributors to health improvements in the last three centuries. During this period, the impact of vaccination on longevity is undeniable, despite the fact that its partial contribution is difficult to distinguish from that of improved hygienic conditions and nutrition, and the discovery of penicillin [3, 4]. All of these combined provided the basis for the so-called "epidemiological transition"[1] in industrialised countries. At the same time, infectious diseases remain the main cause of death in many developing countries. Despite the continuing expansion of the vaccine portfolio, implementing financially sustainable basic vaccination programs in poor countries remains problematic. Though this is not so much an economic as a financial aspect, we will return to this issue in the section "Financing vaccines".

Peculiarities of vaccination and the vaccine market

A number of peculiar characteristics set vaccination apart from other interventions in health care [5]: (1) Since vaccination is (usually) a form of primary prevention, it intervenes in people (often children) who are generally in good health. But unlike most other prevention programs, the interven-

1 Epidemiological transition: transition from infectious and parasitic diseases (in children) as the main causes of death to chronic and degenerative diseases of adulthood. This transition evolved parallel to the demographic transition: transition from high fertility and death rates to low fertility and death rates.

tion itself can cause harm to the vaccine recipient, because in rare cases vaccine-associated adverse events (VAAE) occur. This means that people make trade-offs between risks of vaccine-preventable disease and risks of VAAE. The perception of these risks is quintessential to the individual demand for vaccines (if left to free-market mechanisms), and dominates the influence of other factors such as price [6]. (2) Vaccination not only protects vaccine recipients, but it also reduces exposure of unvaccinated people, due to the reduced circulation of the infectious agent (if the transmission of infection occurs from human to human). This is not always beneficial for public health as the reduced risk of transmission leads to an increased average age at infection (with many "childhood" infections being more severe if contracted in adulthood) [7, 8]. Together with the first characteristic, this means that people generally have an interest in having everyone else vaccinated, but not themselves (or their children). (3) A number of infections can be eradicated in the long run if vaccination efforts are sustained at sufficiently high coverage levels around the world. In other words, sometimes vaccination has the potential of making itself redundant. Choices about eradication are closely linked to the welfare of future generations and societal time preference (see also below) [5, 9].

Since the perceptions outlined above are usually distorted by insufficient or biased information, government intervention (in the form of subsidies, or coercion) is desirable to ensure that vaccine uptake remains optimal. Indeed, as uptake increases the risk of VAAE remains constant, but individuals may perceive it to increase. At the same time the absence of vaccine-preventable disease may create a false sense of security, and lure people into believing that their risk of disease has reduced to zero as well, while this is highly dependent on historical and future rates of exposure and vaccination in the rest of the population.

The vaccine market represents only 1.5% of the global pharmaceutical market, but has high growth potential (estimated at 10–20% per year by various sources, mainly due to new combination, new prophylactic and new therapeutic vaccines) [10]. For a manufacturer, the contribution margins of vaccines are low compared to those of other products in both the developing and industrialised world (due to price and licensing regulations). The few suppliers of vaccines now aim to limit production to the projected global needs in any given year (UNICEF bought about 90% of "traditional" vaccine supply in 2003, compared to about 25% in 1997). Thus the market is very vulnerable to capacity problems: a problem with a single batch of vaccines by a single producer can have severe knock-on effects across the globe. This may also explain why, for some of the old vaccines, the price fluctuates, and has had the tendency to rise over the last 5 years. Close co-operation between demanders (governments or agencies) and suppliers is essential to ensure continued availability at the right time.

Vaccines are supplied under a tiered price system, with 88% of sales volume in developing countries and countries in transition, but 75% of sales

revenue in industrialised countries [10]. It is therefore not surprising that global vaccine manufacturers (with three big producers (GlaxoSmithKline, Aventis and Merck) occupying 77% of the global market) tend to focus on products for the industrialised world.

It is to be expected that more combination vaccines will become available and existing combinations extended. Examples of this include the hexavalent diphteria-tetanus-pertussis-inactivated polio virus – *Haemophilus influenza* type b – hepatitis B (DTP–IPV-Hib–HepB) vaccine, and the quadrivalent measles-mumps-rubella-varicella-zoster (MMRVZ) vaccine. The research and development costs for these vaccines are high due to technical and regulatory complexity. The technicality, the multiple patents and requirements in terms of clinical trials (all demanding great time and money investments) increase barriers to enter the vaccine market. This may lead to more monopolistic behaviour, with risks to supply, choice and price. Clearly, the benefits of combination vaccines are many. For instance, reductions in the number of injections and associated administration costs (including reduced money, time and pain costs for children and their parents), and reduced transmission by contaminated needles benefit recipients and the public health bodies. Free-rider effects (important and not-so-important vaccines can hook up with established vaccines, irrespective of how recipients perceive their importance) and economies of scope[2] benefit manufacturers and perhaps public health bodies. These benefits will have to be traded off *versus* the higher price of combination vaccines.

Because governments, health insurers or agencies (UNICEF, PAHO) typically buy vaccines directly from producers, there is also little diversity on the demand side of the market. All of this implies that there is little competition on both sides of the market and that global societal goals (development and supply of affordable vaccines for poor countries as well as rich countries) are unlikely to be met by relying entirely on free-market mechanisms (particularly since these are hampered by (necessary) regulation with regards to quality control and licensing).

Economic evaluation as an aid to decision-making

Concept

By using economic evaluation we are essentially trying to answer the following questions [5]:

2 Economies of scope are similar to economies of scale, but rather than referring to efficiencies of changes to the scale of the output of a single product, economies of scope refer to efficiencies of changes to the scope of marketing and distributing a range of products (combination vaccines are an example of product building).

1) Is the vaccination program under study worth doing compared to alternative ways of using the same resources? In other words: should the (health care) resources be spent on such a vaccination program, and not on something else?
2) More specifically, if we are deciding to vaccinate against a particular disease, whom should we vaccinate, at which age, with which vaccine and how should the vaccine be delivered and administered in order to deploy our scarce resources in the most efficient way?

Most economic evaluations of vaccination are model-based, because the alternative, empirical analysis, is usually impractical, very time-consuming (for most vaccines it takes decades for the full effects to unfold), very expensive and potentially unethical.

A complete economic evaluation should compare different options for an intervention, in terms of economic costs as well as health consequences. There may be several options to prevent an infectious disease, some of which are mutually exclusive, while others are complementary. The relevant costs and benefits need to be collected for each option, and calculated relative (incremental) to another option. The choice of the reference strategy against which the other options are evaluated can be highly influential for the results of the evaluation. Unless it is a cost-ineffective strategy, current practice is the preferred strategy of reference. When a new vaccine is introduced, the reference strategy is often referred to as "doing nothing" (no vaccination), although in this case "doing nothing" usually means the treatment of cases as they arise, with the corresponding public health measures.

Perspective: costs and benefits for whom?

A generalised distinction between the costs and benefits of vaccination is presented in Table 1. The intervention costs dominate the cost side. These are the costs necessary to implement the vaccination program. Additionally there are costs incurred to receive the vaccine. The benefits of vaccination are the gains in health and the avoided costs. Direct costs can be avoided because less treatment is needed for curing or nursing the disease against which the vaccination program is aimed. Additionally indirect costs can be avoided because vaccination may partly prevent people having to interrupt their normal activities in society because of their illness or the illness of their relatives.

From the health-care payer's point of view, only direct medical costs need to be taken into account. However, from society's viewpoint, indirect non-medical costs are also relevant. Other viewpoints can be those of patients, hospitals, travel clinics, insurance companies, employers, etc. (see Fig. 1). For each of these perspectives different costs and benefits may be

Table 1 – Main costs and benefits of vaccination

Costs	Benefits
Direct costs (mainly intervention costs)	Avoided direct costs (mainly treatment costs)
Indirect costs (mainly time lost to receive vaccination)	Avoided indirect costs (mainly time gained by avoiding illness)
	Health gains (physical and psychological)

Source: [5]

relevant. This implies that it is possible for an intervention to be relatively cost-effective for one party involved, while it is not for another.

Different cost categories are listed in Table 2. The listings *in italics* are often not taken into account, because they can be relatively small in comparison to the other costs and/or because they are difficult to estimate. Sometimes their inclusion is not relevant to express the viewpoint of the analysis. However, if they are relevant for the viewpoint of the analysis, their impact on the results could be tested in a sensitivity analysis and their existence should be mentioned when the results are presented.

Some diseases affect expectations and behaviour beyond one degree of separation from the pathogen. For instance the global impact of the SARS outbreak in 2003 was modest in disease burden (8098 probable cases, 774 deaths) and associated health-care costs, but it had an impressive impact on the global economy (US$30–100 bn, or $3–10m per case) in macro-economic terms (when the impact on consumption and investments are considered) [11, 12]. A similar situation could arise for pandemic influenza, or any other disease that affects risk perceptions of consumers and investors (e.g., variant Creutzfeld Jacobs Disease). However, for most currently vaccine-preventable diseases, micro-economic evaluation would provide an appropriate analytical framework, preferably adopting a societal perspective. In reality, decisions about universal vaccination are often taken from the perspective of the National Health Service (NHS) or the Ministry of Health and at best from the health-care payer's perspective (which in addition to the NHS costs also includes direct co-insurance and co-payments by the patient). Indeed, decision-makers in health care tend to focus primarily on direct costs since these are most indicative of their immediate budgets, even if their decision has bearings on society at large.

Opportunity, average and marginal costs

When it comes to estimating unit costs or prices, it should be noted that costs in an economic sense are opportunity costs: they represent a sacrifice of the next best alternative application [13]. This entails that costs in an eco-

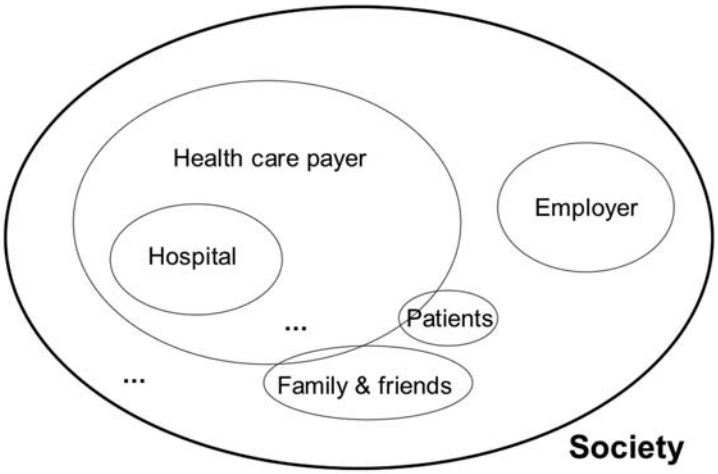

Figure 1. Perspective: different costs (and effects) may be relevant for different analytical viewpoints

nomic sense are not necessarily the same as financial expenditures, and that they can also represent goods and services that are not expressed in monetary terms. However, market prices are often used as a proxy. If particular goods and services are not traded on a market, ("shadow -") prices of a similar activity can be used instead. For example, work of volunteers can be approximated by wages of unskilled labour. Similarly, patients' leisure time could be based on average earnings or average overtime earnings. Average costs per unit of output are the total costs of producing a quantity divided by that quantity. Marginal costs constitute the additional costs of producing one additional unit of output. Since decisions are made at the margin, marginal costs should be used where they are substantially different from average costs [13]. For vaccination, this distinction is most relevant for estimating the costs of the program [5]. The costs of adding a particular vaccine to the existing program depends on how well the schedule of the new vaccine fits in with the other schedules, whether specific precautions need to be taken, whether potential vaccinees need to be screened prior to vaccination or whether a specific target group is envisaged. The costs that are most heavily affected by adding a new vaccine to the existing program are the variable costs of the program (time spent per vaccinee, number of vaccines bought, etc.), whereas the influence on the fixed intervention costs (buildings, general equipment, etc.) is usually small (unless a new vaccine requires a substantially different infrastructure in terms of storage and transport). A good example of this is provided by Hall et al. who examined the immunisation program in The Gambia (more recently these results were confirmed by a similar analysis in Addis Ababa, Ethiopia) [14, 15]. They found that the additional costs of adding hepatitis B vaccine to the existing Expanded

Table 2. Cost categories in an economic evaluation of a vaccination programme

Direct costs:	- Treatment costs directly related to medical consumption (consultations, medication, diagnostic tests, surgery, etc.) for diagnosis and treatment of the diseases against which the vaccine offers protection. - Intervention costs, directly related to the implementation of the vaccination program. These consist of administration costs (consultations, needles, syringes, screening, information campaign, cold chain, infrastructure (buildings, vehicles, etc.), training, etc.) and vaccine purchasing costs (based on the number of required doses, including wastage). Treatment costs for vaccine associated adverse events (VAAE) are often also included here. - *Personal direct costs (transport, home treatment, special education, etc.)*
Indirect costs:	- Costs of time losses (unpaid and paid productive time (and arguably leisure time) of patients and their caretakers) - *Unrelated future health care costs (future health care costs incurred during prolonged lifetime)* - *Unrelated future non-health care costs (future costs of living borne by society during prolonged lifetime)*
Intangible costs:	- *Costs of anxiety, pain and suffering*

Source: [5]

Program on Immunisation (EPI) vaccines (measles, polio, DTP and Bacille Calmette-Guérin (BCG)), would be for 94% recurrent costs (of which 87% for purchasing hepatitis B vaccine (hepB)). Still, the introduction of a new expensive vaccine could more than double the costs of the program in some countries because of its sheer price compared to other vaccines in the program.

Valuing health outcomes: different types of economic evaluations

The main objective of vaccination is to prevent disease. The most important benefits from a public health point of view are therefore the health gains (see Tab. 1). These are both physical (avoiding illness, suffering, mortality, etc.), and psychological (avoiding distress, anxiety, etc.). Specific vaccine-related psychological health gains include the general feeling of well-being and security of vaccine recipients from knowing that they are protected against disease. This could evidently lead to behavioural changes (e.g., a vaccine against HIV/AIDS could have a large influence upon the sexual behaviour of vaccine recipients).

The valuation of health outcomes has far-reaching consequences for the methodology and study design of applied analyses. Generally, a distinction is made between four different methods, depending on the way in which health gains are measured [13].

A cost-minimisation analysis compares the costs of equally effective alternatives, without quantifying the health gains. It differs from a pure cost

analysis in that there is always more than one option analysed and that the effectiveness of the different alternatives is known to be equal.

In a cost-effectiveness analysis, health gains are measured in one-dimensional natural units (e.g., infections prevented, hospitalisations prevented, deaths averted, life-years gained…), implying that only one aspect of effectiveness is considered (e.g., postponing the time of death) and other related aspects are not (e.g., the quality of life). The results of cost-effectiveness analyses (CEA) are usually presented as a ratio. A Cost-Effectiveness Ratio (CER) is a measure of the incremental costs, which are necessary to obtain one unit of a health gain by implementing a strategy j instead of a strategy i (expressed in incremental costs per life-year gained, incremental costs per infection prevented, etc.).

$$\text{CER} = \frac{\text{Costs with strategy j} - \text{Costs with strategy i}}{\text{Effects with strategy j} - \text{Effects with strategy i}}$$

The lower the ratio, the more efficiently strategy j gains health compared to strategy i.

The units in which health gains are expressed should represent the final results or clinical endpoints of an intervention as adequately as possible, in order to enable comparison between different interventions [16]. If, hypothetically, the cost-effectiveness of hepatitis B vaccination were $50 per infection prevented, whereas Hib vaccination is estimated at $50,000 per infection prevented, it is wrong to conclude that hepatitis B vaccination is more cost-effective (because it is less costly to prevent one infection). To make that judgement, the avoided effects would need to be expressed in a more comparable endpoint, like life-years saved. To make the comparison even more relevant, different health states should be weighed by their quality (scaled from 0 (meaning death) to 1 (meaning perfect health)). This approach is used in cost-utility analysis (CUA), where health gains are measured in Quality-Adjusted Life-Years (QALYs) saved or another combined measure of morbidity and mortality (e.g., Disability-Adjusted Life-Years (DALY)). A Cost-Utility Ratio (CUR) is similar to a CER, except that the denominator contains the difference in QALYs (or DALYs), instead of the difference in natural units, such as cases avoided or life-years gained. The main advantage of CUA over CEA is that it allows comparison of very different health-care interventions, for instance, those that predominantly extend lives (e.g., flu vaccination of the elderly) with those that improve the quality of life (e.g., drugs against erectile dysfunction).

In Cost-Benefit Analysis (CBA), the health gains are converted into monetary units, which, in theory, allows the many dimensions that are associated with an improvement in health status (over and above the length and health-related quality of life) to be included. There are benefits beyond the health outcomes such as information, caring, regret, anxiety reduction, communication and process utility (benefits from health-care use). Further-

more, option value (i.e., benefits derived from needing care in the future) and non-use value (i.e., externalities related to caring for the health of others) can also be (potentially) elicited [17]. The results of a CBA can be presented as the difference between costs and benefits (the net costs (or net savings)) or as a ratio. The benefit-cost ratio (BCR) expresses to which extent an investment in an intervention can be recovered by the consequences of that intervention (expressed as a unitless number or a %).

Cost-benefit analysis allows for comparisons between totally different projects in society (e.g., comparing a vaccination campaign with the construction of a new bridge). When budgets are very limited and many urgent interventions compete, as in developing countries, such cross-sector comparisons may actually be used in practice. Clearly, the potential of CBA to make such comparisons possible is a major advantage to aid decision-making.

The strength of CBA in theory, i.e., the explicit monetary valuation of health gains, has up till now been also its weakness in practical decision-making. In theory it seems preferable that the valuation of health gains (and of life) is done in an explicit, transparent and representative way as in CBA, instead of the implicit, inconsistent and arbitrary way it is often done in today's decision-making. However, in a health-care environment the monetary valuation of health (and particularly of life) is often rejected on an emotional basis[3] [18]. Additionally, economists have few credible arguments to counter these objections, as the current methods which place a monetary value on health (human-capital and willingness-to-pay methods) can hardly be called consistent and reliable in practice [19–21]. In view of this, most economic evaluations in health care are based on CEA or CUA. The literature on these has increased exponentially since the 1980s, for vaccines at least as much as for other interventions in health care, underlining the importance of a sound theoretical framework for these analyses [17].

Time preference

Individuals (and societies), in general, prefer to receive benefits as early as possible and incur costs as late as possible. This so-called time preference means that the same amount of wealth or health would have a different value to a decision-maker in the present, if this amount is gained at different points in time. Note that time preference has nothing to do with inflation. A vaccination program is an investment made in the present (i.e., the costs of buying and administering vaccines) to gain benefits spread out over the future (i.e., avoided costs of treatment, avoided morbidity and mortali-

3 As noted by Mooney [18]: "To be trained in medicine, nursing or one of the other 'sharp end' disciplines and then be faced with some hard-nosed, cold-blooded economist placing money values on human life and suffering is anathema to many."

ty). Discounting is a technique by which future events (e.g., costs and health outcomes) are valued less the further in the future they arise. The degree to which they are valued less is determined by the discount rate (frequently assumed to be constant through time): the higher the rate the less future benefits and costs are valued. Although there is general agreement on the discounting of costs, the arguments for discounting non-monetised health outcomes are contradictory [5]. Discounting costs without discounting benefits leads, amongst others, to the paradoxical situation that any eradication program will yield infinite benefits [13]. This would imply that all current resources should be spent on research of eradicable diseases, and the implementation of eradication programs, and not a single penny on cure. Such paradoxes, and the observation that individuals generally have a positive discount rate for health, clearly indicates that health too should be discounted at a positive rate. But there is no general agreement on how the discount rate for health should compare to that of wealth. There are arguments to apply an equal discount rate to both costs and health effects [22, 23]. The underpinnings and relevance of these are questionable, so that a lower discount rate for health effects than for costs has also been proposed [24, 25]. Because of the very long time spans over which benefits accrue, the analysis of most vaccination programs is very sensitive to discounting (of costs as well as health effects). Nonetheless, this is no cause for a different approach to discounting for vaccination. Still, further empirical research is needed to strengthen or to change the basis for conventional discount rates (mostly 3%, or 5%) and discount models (mostly stationary) [5]. A slight decrease in discount rate (from, say, 5% to 3%) could change the cost-effectiveness of some vaccination programs from unattractive to attractive. Also, it is likely that time preference in developing countries is substantially different (i.e., higher) from that in industrialised countries, particularly for those countries that have decreasing health (e.g., life-expectancy due to HIV/AIDS) or wealth (e.g., real GDP) expectations [5].

Decision-making: are vaccines a good buy?

In theory, decisions are made by interpreting the results of economic evaluation as follows. In Figure 2 a new program is plotted in terms of costs and effectiveness *versus* the reference strategy in the origin. If the new program is less costly and more effective than the reference, then the new program (a "dominant" strategy) should be implemented. Likewise, if the new program is more costly and less effective than the reference, it should be rejected. In the other quadrants the decision is more complex, because it depends on a value judgement. If the incremental CER (or CUR) is smaller than a given willingness to pay (or threshold cost-effectiveness criterion), "K", it would be acceptable. The question then is, how to determine K? This could be determined by social debate or by comparing it to what is widely accept-

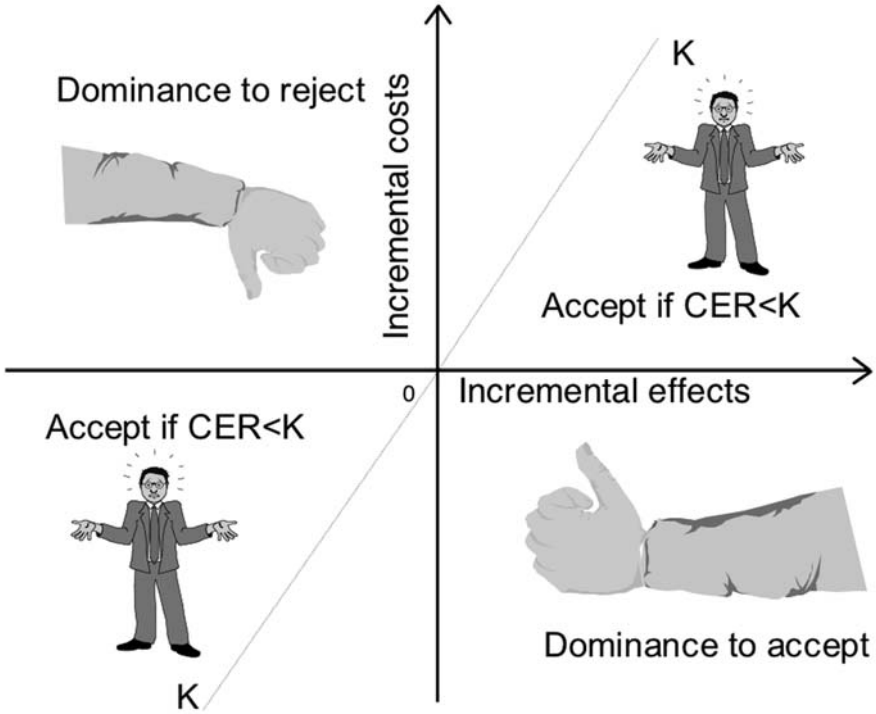

Figure 2. The cost-effectiveness plane. CER: Cost-effectiveness ratio, i.e., incremental costs divided by incremental effects; K = willingness to pay, or a cost-effectiveness ratio of acceptable magnitude. All points on a line in this plane have identical cost-effectiveness ratios.

ed in practice. The most widely cited K in industrialised countries is $50,000 per QALY gained. There may also be a grey zone for K in which some interventions are implemented and others are not (e.g., between $40,000 per QALY gained and $100,000 per QALY gained), whereas under and above that grey zone all and none of the interventions are implemented, respectively. However, the greater the analytical uncertainty and the burden of disease, the more decisions are likely to deviate from such clear cut-off practices [26]. Different societies should have a different willingness to pay, though there are few instances in which societies (or their decision-makers) have tried to determine what the appropriate value of K is. The World Bank has suggested using GNP per capita as a benchmark for K. Note that in CBA, K has already been given an explicit value.

In league tables, many vaccination programs rank with the most cost-effective interventions in health care in industrialised countries [5, 27–29]. It is tempting to try and estimate the global historical value of vaccination. However, due to scarcity of data in most parts of the world such an exercise

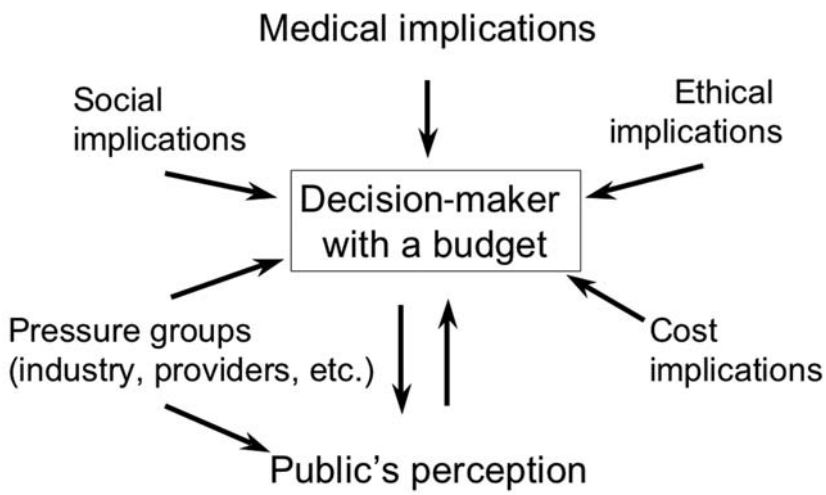

Figure 3. Factors influencing decision-making in practice

would be, by necessity, extremely crude. It seems clear, though, that the smallpox eradication program and the establishment of the EPI have generated enormous benefits, not only by directly protecting against important vaccine-preventable diseases, but also by providing opportunities for health education and infrastructure in developing countries [30]. Yet the associated disease reduction in smallpox, measles and tetanus alone is bound to have been a cost-saving enterprise around the world (i.e., in the lower right quadrant of Fig. 2), currently averting over 8 million deaths *per annum*, compared to a "never having vaccinations" situation. However, when we are making choices today, we have to consider what additional benefits we will achieve by making additional investments, and this is bound to vary between countries at different stages of economic development, different epidemiologies of disease, and different historical vaccine-uptake levels. Hence data from one country cannot always be simply extrapolated to another.

In practice, there are many factors that come into play when decisions are made about new health-care interventions (see Fig. 3). In a democracy, a decision-maker receives a temporary, renewable mandate from the public to allocate a given budget. That person is well aware of the public perceptions of public health problems, and the impact of decisions thereon. At the same time, pressure groups may try to influence decision-makers or the public's perception. These pressure groups have vested interests in the decisions (be it as sellers of vaccines, or sellers of services for the cure of vaccine-preventable diseases). Societal goals with regards to the decision can only be met by considering its medical, social, ethical and cost implications. The theoretical foundation of economic evaluation (so-called "Pareto opti-

mality") addresses efficiency, without concern for distributional aspects (equity). Therefore, economic evaluation combines the medical/epidemiological and cost implications, but does not consider the social and ethical implications depicted in Figure 3 (though in CBA these aspects could theoretically be included, if a willingness-to-pay approach is used, and it is possible to weight quality-of-life gains to help achieve equity goals, as is commonly performed in DALYs). Therefore economic evaluation should be seen as an additional type of analysis that cannot stand on its own in its current form (it is an aid to decision-making, not a decision-maker in itself). At the same time, ideally, the influence of pressure groups, and the public's perceptions (rather than the public's true preferences) should be minimised in this process. It is noteworthy that most vaccination programs are likely to be equitable according to prevailing theories of justice [5]. Indeed, an analysis for Bangladesh indicated that socio-economic inequalities in mortality of under-5-year-olds were eliminated by measles vaccination [31].

Financing vaccines

In the past, vaccination interests of poor and wealthy nations seemed more in tune than today. Moreover, the research and development costs of the new generation of vaccines, based on biotechnology, are greater and the regulatory hurdles higher, meaning that new vaccines are much more expensive than the basic package of "traditional" vaccines. The first new expensive vaccine for global use was the hepatitis B vaccine, which became available in 1981. The main reason why it was not immediately included in universal vaccination programs was its price, because initially the hepatitis B vaccine cost more than the other six EPI vaccines put together.

With the advent of more expensive vaccines, the introduction of a new vaccine is not as straightforward as it used to be in the industrialised world. In contrast to some of the "older" vaccines (e.g., measles, pertussis), newer vaccines may not result in net savings to the health-care system. Nonetheless, if considered desirable, industrialised countries have no difficulty in financing the introduction of new vaccines, and ensuring the continuing uptake of old ones. For developing countries, the main difficulty is not so much to determine whether it would be cost-effective to introduce a vaccine, but to ensure that the introduction is financially sustainable. When external donors sponsor vaccination programs the sustainability takes the form of a partnership with shared responsibility and the promise by the receiver of the financing to create the conditions to become self-sufficient in the long run, either alone or by attracting further external funding.

Global immunisation efforts came under pressure as the EPI, which was launched in the 1970s as a way of building on the success of the smallpox eradication program, lost its momentum in the 1990s, and failed to attain the year 2000 goal of 90% global vaccination coverage. Indeed, global child-

hood immunisation coverage against the six main target diseases (polio, DTP, measles and tuberculosis), which was less than 5% in 1974, decreased from about 80% in 1990 to 75% in 2000 [32]. Coverage for the complete schedule of DTP remains well below 75% in tens of developing countries, mostly in sub-Saharan Africa. These countries are traditionally bottlenecks in the EPI because of great financial constraints, the evolution of the HIV epidemic, logistical difficulties, poor governance and general socio-economic conditions (sometimes aggravated by war). As these factors evolved unfavourably in the 1990s, international alliances shifted their efforts from reducing general global inequalities in health ("Health for All") to more selective strategies, like the polio eradication program and the introduction of new and improved vaccines.

The discrepancy between the developing and the industrialised world is likely to become greater, as private vaccine development focuses primarily on diseases that affect the wealthy. Indeed, only about 1% of world drug sales is for African countries. It has been estimated that of all expenditures on health research (over $70 billion per year), 90% is for diseases that affect 10% of the world's population [33].

Using recent examples, Kaddar et al. assert that financing vaccination should be affordable by all countries, at least for the basic vaccines [34]. The cost of fully immunizing a child with the basic vaccines is $5 to $25, which typically represents 5% to 10% of public health expenditures, <$0.4 *per capita* or about 0.1% of GDP. Most vaccination costs are fixed costs of personnel and infrastructure, and the marginal costs of an additional vaccine may often be bearable for the domestic budget (though still highly dependent on vaccine price). As the 20th century drew to a close, the landscape of external vaccine financing underwent dramatic changes with the inception of the Global Alliance for Vaccines and Immunization (GAVI) and the Vaccine Fund, with the aims to stimulate research and development for developing world problems, strengthen immunization systems, and promote and support the introduction of new and underused vaccines. GAVI is an alliance of financiers (development banks, aid agencies, foundations), agencies (UNICEF, WHO), vaccine developers and manufacturers, as well as developing country governments, whereas the Vaccine Fund manages private financial resources, such as those from the Bill &Melinda Gates Foundation, and public contributions from a small number of wealthy countries.

Conclusion

The first generation of vaccines, such as measles and oral polio vaccines, was used against common and serious childhood diseases afflicting all countries in the world. Few of these vaccination programs were subject to economic analysis before introduction, and for good reason: the benefits

were obvious and the costs low. Indeed, they were probably amongst the most effective and cost-effective public health programs of the 20th century. This is no longer necessarily the case with new vaccine introductions. New vaccines are generally higher priced and unlikely to fall in price to the level of the first generation of vaccines. Furthermore, they are often aimed at less common or less serious diseases (particularly in the industrialised world). Thus, whether these vaccines are worth introducing is less clear. Vaccine financing has recently changed with important initiatives stimulating development and use of vaccines for the developing world. These are to be welcomed as they may further alleviate the disease burden in developing countries at affordable cost, correct market imperfections with regard to research and development, and reduce inequalities in health. Nonetheless, the introduction of new vaccines demands cautious planning. If it comes at the expense of the uptake of the first generation of vaccines, it may have a detrimental influence on the effectiveness and cost-effectiveness of the whole program. In view of all these developments, the role of economic evaluation in vaccine program design is only likely to increase in the future.

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Private hand

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How and why vaccines are made

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Introduction

Vaccines to prevent disease remain the ultimate goals of medicine, following the proverb that says in many languages "Better to prevent than to cure." Certainly, the record of vaccines has been one of exemplary success in reducing, eliminating and even eradicating infectious diseases. Yet there is not a general appreciation of the difficulties that attend the development of vaccines, difficulties that increase with each passing year. This chapter attempts to give a perspective on the process of vaccine development: why a target is chosen, how the vaccine is developed for licensure, and what is involved in the modern production of a biological vaccine for use in humans.

Aside from the medical and humanitarian advantages of vaccination, the cost-benefit ratio is extremely favorable, as shown in Figure 1 [1].

The vaccine industry

Manufacture of vaccines is highly concentrated from an economic point of view, with five companies accounting for more than 80% of the market: Aventis Pasteur, Chiron, Glaxo SmithKline, Merck and Wyeth (Chryssomalis G, Hardy C, Sanofi Pasteur, personal communication; Fig. 2). These five manufacturers, and indeed most of the manufacturers in the second rank, are based in industrialized countries. However, there are many other manufacturers supplying limited ranges of vaccines or having sales in limited regions (Tab. 1). The largest part of the vaccine market is in North America, with Europe second and the rest of the world only accounting for about 15%. However, in terms of numbers of doses used, manufacturers from the developing world play a major role, particularly the Serum Institute of India in recent years.

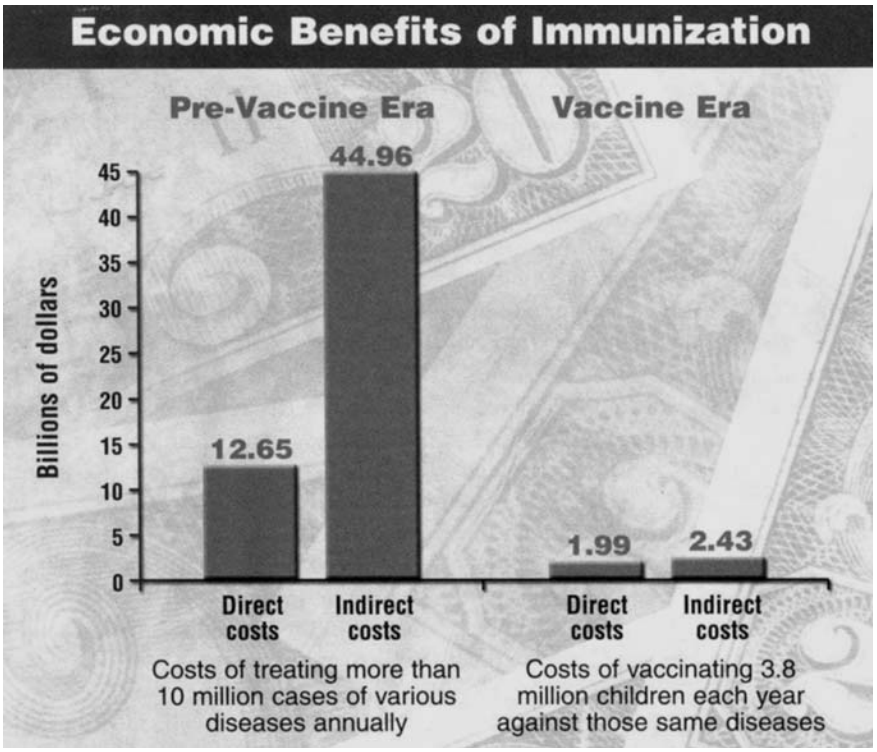

Figure 1. If there were no immunization in the United States, 33,494 deaths and 10,541,569 cases of diphtheria, tetanus, pertussis, polio, measles, mumps, rubella, congenital rubella syndrome, Hib and hepatitis B infection could be expected each year.

Although all of the major vaccine manufacturers are part of larger pharmaceutical corporations, vaccines are not the same as drugs. Vaccines are given to healthy people rather than to ill people, low efficacy is much less acceptable, infants are the primary targets, and they are given only a few times in life. Moreover, they are supposed to be cheap, which is inconsistent with modern quality control. The latter point is all the more problematic as the total vaccine market is only about 1% of the pharmaceutical market, and thus not attractive to investors.

The main products that generate income are Hepatitis A and B, MMR and flu vaccines. Flu vaccines are profitable because of high volume, whereas the hepatitis vaccines and MMR elicit high prices in industrialized countries. More recently, pneumococcal conjugate vaccine has turned into a "blockbuster". As newer vaccines are much more costly than older ones, for reasons that will be made clear below, it is likely that future vaccines will not be cheap.

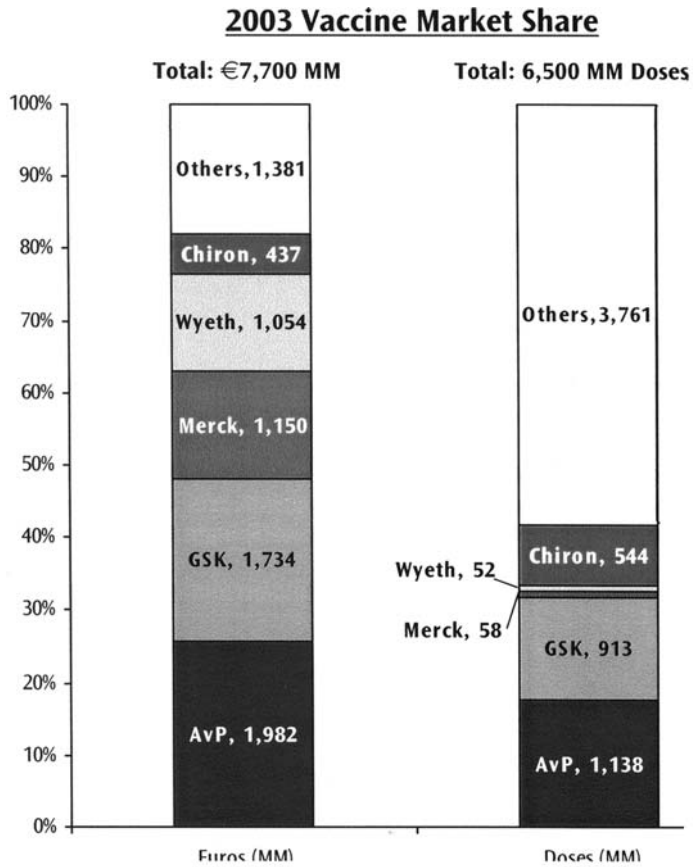

Figure 2. The vaccine market analyzed by income generated (million Euros) and by number of doses sold (million doses). GSK, GlaxoSmithKline, AvP, Aventis Pasteur, now Sanofi Pasteur.

Why does industry develop certain vaccines?

The selection by industry of which vaccines to develop is a complex one, involving many different factors, listed in Table 2. As industry must make a profit in order to satisfy investors, the size of a potential market is a key factor. Table 3 lists the often imprecise means by which market size is gauged. Demand from consumers is of course important, but difficult to measure. It was assumed that a high demand for a Lyme disease vaccine existed in the Northeast United States, but when the vaccine actually was commercialized, demand was feeble. In contrast, consumer demand propelled the development of less reactogenic acellular pertussis vaccines.

Demand from governmental authorities is more reliable, as ultimately they must pay for a vaccine. The request from United Kingdom authorities

Table 1. Vaccine manufacturers

Large market share:	Sanofi Pasteur (France, Canada, USA) Chiron (Italy, Germany, USA) GlaxoSmithKline (Belgium, USA) Merck (USA) Wyeth-Lederle (USA)
Smaller market share:	Baxter (Austria) Berna (Switzerland) Commonwealth Serum Labs (Australia) Serum Institute of India
Limited range:	Bioport (USA) Dynport (USA) Korean Green Cross Medimmune (USA) North American Biologicals (USA) RIVM (Netherlands) Powderject (UK) Shires (Canada) Solvay Statens Serum Institute (DK) Swedish Bact. Lab Acambis (UK, USA) Vaxgen (USA)
Regional:	Brazil China Cuba India Indonesia Iran Japan Russia South Africa Thailand

for a conjugated meningococcal vaccine drove several companies to develop one.

Epidemiological data, such as those collected on invasive pneumococcal infections, were a great stimulus for the development of protein-conjugated polysaccharide vaccines for infants. Epidemiological data are also often the basis for expert opinion. Experts serve as "champions" for particular vaccines. For example, it is unlikely a live mumps vaccine would have been developed if Maurice Hilleman at Merck had not insisted on it.

Finally, there is a good deal of guesswork involved in marketing predictions. Few would have imagined that hepatitis B vaccine would become a standard pediatric vaccine at the time when homosexuals and recipients of blood transfusions were considered the primary targets.

However, market size is far from the only consideration. Vaccine manufacturers respond to public health need, although unfortunately they some-

Table 2. Reasons why vaccine manufacturers launch a development program

1	Market
2	Public health interest
3	Technical feasibility
4	Intellectual property
5	Fit with other vaccines

Table 3. How market is determined

1	Demand from consumers in developed countries; e.g., Lyme disease, acellular pertussis
2	Demand from authorities in developed countries; e.g., Meningitis C
3	Epidemiological data; e.g., pneumococcal conjugate
4	Expert opinion; e.g., mumps
5	Guesses, buttressed by precise but inaccurate data; e.g., hepatitis B

times fail to receive useful advice from public health authorities. On the other hand, documents such as the Institute of Medicine's priority classification for the development of new vaccines are very helpful.

A key factor in the choice of vaccines for development is technical feasibility. By and large, the vaccine industry does not do basic research, but rather develops findings made in academic or biotech laboratories. If the latter have not provided a practical means of making a vaccine, manufacturers are loath to embark on a costly vaccine development effort. Moreover, academicians frequently do not understand how an idea that works in their laboratories on a small scale must be developed in a practical way, which at times may be impossible. The problem is usually scale-up, a process fraught with difficulties. Also, an experiment that works in mice may fail in humans, or as is sometimes said, "mice lie, or at least exaggerate."

Availability of intellectual property is another important factor, and unfortunately is still a quagmire [2]. Patent protection blocked the production of hepatitis B vaccine by multiple manufacturers for many years. On the other hand, the licensure of the Lyme disease OspA vaccine was made possible by a royalty agreement between two manufacturers who both held important intellectual property.

Lastly, a company may decide for or against a vaccine based on its fit with other vaccines the company produces. This becomes most obvious with regard to combinations: for example, if a company has no access to a pneumococcal conjugate vaccine, it may not wish to develop an Hib vaccine to prevent meningitis.

The strategies of vaccine development

Vaccine development has evolved greatly in recent years. Attenuation of living pathogens has been a major strategy, and continues to be so.

Table 4. Some newer strategies for vaccine development starting from information on the microbial genome (DNA, cDNA, or RNA)

Strategy	Examples
Recombinant protein production	Hepatitis B S_{Ag}, pertussis toxin, Lyme outer-surface--protein A
Live recombinants	Dengue, parainfluenza, tuberculosis
Replication – defective particles	Human papilloma virus, herpes simplex virus
Alpha virus replicons	Human immunodeficiency virus, hemorrhagic fevers
"Naked" DNA Plasmid	Hepatitis B
Recombinant vectors	Cytomegalovirus, human immunodeficiency virus
Prime boost using DNA and/or vectors	HIV, malaria
Reverse genetics	Influenza, parainfluenza, respiratory syncytial virus

Similarly, inactivation of whole bacteria or viruses remains a useful technique in certain cases, but recently the tendency has been to break down the organism to extract the protective antigen. Thus, the use of purified bacterial capsular polysaccharides, protein subunits, and detoxified toxins is seen. Reassortment of genome segments by coinfection with wild and attenuated viruses in tissue culture has been used for some time in the development of killed influenza vaccine, and more recently for the development of live influenza and live rotavirus vaccines.

With the advent of genetic engineering, many new strategies have become available, listed in Table 4 [3–5]. They have not only made vaccine development more feasible, but in a sense they have changed the definition of a vaccine. In 2004, a vaccine might be defined as a protein, polysaccharide, nucleic acid, living vector or infectious agent, that when inoculated or administered to the mucosa, induces antibodies and/or cellular immune responses specific to a pathogen that protect against the pathogen or suppress its chronic replication.

Recombinants of yeast, bacteria or animal cells are now used to produce proteins of many descriptions, most notably the hepatitis B surface antigen. Living recombinants themselves serve to carry genes from multiple related viruses, such as the envelope genes for four dengue serotypes, all carried on one attenuated dengue serotype. Replication-defective particles carrying no RNA or DNA can often be constructed from one or a few viral proteins. Alphavirus replicons are a special case of pseudoparticles, in that expression of foreign antigens during abortive replication can immunize a host. "Naked" DNA plasmids stimulate immune responses when injected, particularly cellular responses.

A major field in vaccinology is currently the use of vectors, which are attenuated bacteria or viruses into the genome of which are inserted genes that code for vaccine antigens. Examples of vectors are poxviruses like vaccinia, adenoviruses, and BCG. The expression of the inserted gene is often sufficient to stimulate both antibody and cellular responses, particularly the latter. Although vectors may be useful in themselves, they are frequently

employed as primes or boosts in a so-called prime-boost configuration. For example, a vaccinee may be primed with a DNA plasmid and boosted with an adenovirus vector, both carrying the same genes from a pathogen.

Reverse genetics refers to the directed mutation of microbial genomes with subsequent examination of phenotype, which allows for rapid attenuation and identification of desirable mutations.

The process of vaccine development

The road to vaccine development is outlined in Table 5. As stated above, the basic ideas are likely to come from outside of industry, but at a certain point a vaccine can only advance with the help of industry scientists. Once a vaccine lot is produced for clinical trials, it can pass from the preclinical phase, which is conducted in animals and *in vitro* (Tab. 6); to phase 1, in which basic safety and immunogenicity are studied (Tab. 7); to phase 2, in which the dose, schedule and other properties of the vaccine are defined (Tab. 8); to phase 3, which defines safety, efficacy and consistency of manufacture (Tab. 9); and finally to phase 4, which measures safety and effectiveness after licensure by observation of large numbers of vaccinees.

Table 5. The road to vaccine development

Academic/biotech	
1	Identify the mechanism of natural protection against a pathogen
2	Isolate the antigen(s) responsible for the protection
3	Show in animals that the vaccine protects
4	Find the best method to present the antigen (live, killed, subunit)
Industry	
5	Increase yield and purity
6	Develop a process for large-scale production
7	Show the safety of the antigen in animals
8	Produce a lot under GMP
9	Proceed to clinical development

Table 6. Preclinical development

1	Define vaccine characteristics: (nature, chemical definition)
2	Follow good manufacturing practices:
	Write clear definition of a reproducible process
	Quality control: Show homogeneity from batch to batch.
	Show microbiological sterility
3	Animal tests
	Safety
	Immunogenicity
	Efficacy, if animal model is available

Table 7. Phase I Clinical development

- Safety in healthy adult volunteers (safe = absence of serious or unexpected effects)
- Usually fewer than 50 subjects not exposed to the natural disease
- Verify immunogenicity in volunteers
- Open uncontrolled study or randomized controlled study
- Maximal dose
- One or several dose vaccination schedules

Table 8. Phase II Clinical development

- Randomized, double-blind, controlled studies (control = reference vaccine or placebo) 100–200 subjects per group
- Cascade of studies to determine:
  - optimal dose (dose-response)
  - administration route
  - vaccination schedule (including the need for booster dose)
  - possible association with other vaccines (lack of interference, no acceptable increase of side-effects)
  - response in presence of pre-existing antibodies (natural or maternal antibodies)
  - safety: tolerability (incidence, duration and severity of side-effects)
  - immunogenicity: antibody response by standardized tests (geometric mean titer, AB kinetics and persistence, rate of responders, seroconversion rate, AB functionality)

Phase 2b (optional)
Efficacy study in high-risk population – small numbers, low statistical power

The preclinical phase includes the definition of the potential vaccine and the demonstration of likely safety and immunogenicity in animals. If there is an adequate animal model, efficacy will also be studied. A clinical lot is then produced under Good Manufacturing Processes.

Phase 1 involves demonstration of basic safety and immunogenicity in a limited number of healthy adult volunteers. A small number of control subjects may also be included. Dosage may also be studied in this phase, although more usually that occurs in phase 2.

Phase 2 comprises an enlargement of data on safety and immunogenicity, definition of optimal dose and schedule, and a variety of intense immunological tests of antibody and cellular responses. If the attack rate of the disease for which the vaccine is being developed is high enough, a phase 2b efficacy study may be organized. This is an effort to verify that the vaccine is likely to be efficacious in more extensive tests, although the statistical power of the phase 2b demonstration will be low.

Phase 3 involves three important considerations: verification of safety in 10,000 to 50,000 individuals, efficacy in a double-blind, placebo-controlled, randomized study with good statistical power, and demonstration of consistency between lots. The latter usually involves clinical tests of at least three lots for statistically similar immunogenicity.

Table 9. Phase III Clinical development

- Population: target population (susceptible, exposed)
- Safety profile in large numbers (10–50,000)
- Measurement of the protective effect of the vaccine
  - randomized, double-blind and controlled study for "absolute" efficacy; when controlled with a reference vaccine, obtain "relative" efficacy
  - main evaluation criterion: case of disease

Consistency studies
- test 3 serial lots for equivalent
  - safety
  - immunogenicity
  - stability

Table 10. Phase IV Clinical development (post-licensing)

- Safety in large numbers (millions) of unselected vaccinees
- Effectiveness in actual use (vs un-randomized non-vaccinated)
- Duration of protection

Not surprisingly, the total process is long. The average time from conception to licensing of a vaccine was formerly about 10 years. At this point, 15 years is a more realistic figure.

Nevertheless, the process does not finish there. Phase 4 post-licensing studies are now mandatory. They confirm extent and duration of effectiveness in actual use, but also enable collection of more extensive information on adverse reactions (Tab. 10).

Vaccine production

The modern production of vaccines is a complex and tedious process. Regulatory bodies demand that biological vaccines come as close as possible to drugs with regard to purity and consistency, which is a tall order. Safety becomes a paramount concern, and quality control procedures are designed to exclude chemical or microbial contamination.

Traditional (non-recombinant) vaccine production begins with growth of the bacteria or viruses that cause the disease, followed by inactivation of the organism and/or detoxification of the bacterial toxins (e.g., tetanus), and purification of the cell or a component of the cell. Early vaccines were simply grown on media that supported good growth of the bacterium or propagation of the virus. Complex media including ingredients such as meat extracts and yeast extracts were common components and yielded good bacterial growth. Embryonated chicken eggs still prove to be a productive

method for growing a number of viruses, including most importantly influenza virus. However, most viral vaccines are made in cell culture.

As drug guidelines were applied increasingly to vaccines, better characterization of the vaccines followed. The complex media used in early vaccines were not well defined, as the natural variations in meat, yeast, etc., translated into variable media composition, and hence variable yield and potency. These variations proved to be critical, causing vaccine manufacturers to screen multiple lots of the media ingredients to find some appropriate for manufacturing. Without clear definition of the nutritional requirements for each organism, the appropriate media composition was often difficult to develop. Likewise, for some vaccines whole bacterial cells were replaced by including only the cell components that were linked to protection (e.g., acellular pertussis vaccines are replacing whole-cell vaccines). These were considered new vaccines as new disruption/purification processes were developed and therefore they were submitted to clinical tests for safety and efficacy.

The current manufacture of influenza vaccine proves to be an interesting exercise in logistics. Each year, based on epidemiology of circulating strains, various health organizations worldwide select the virus strains thought to be the greatest threat for the coming year. Generally between February to April in the Northern hemisphere, these strains are identified and manufacturers then race to develop virus seeds from these strains and quickly inoculate many hundred thousand eggs per day to produce sufficient vaccine by the immunization season that fall. Earlier selection of strains could risk a poor choice for the vaccine, while a later choice would jeopardize a sufficient supply. Some firms make educated guesses and begin to manufacture before the official selection of the strains as they know they cannot manufacture enough product otherwise. A poor choice of strains by the manufacturer could have a serious financial impact.

The logistics of influenza production include management of the chicken farms to produce sufficient quantities of hens and cockerels to produce the embryonated eggs. As these eggs will be used in manufacture of a sterile vaccine, control of the farms, incubators, and delivery network is paramount. The entire process must also be protected by numerous security measures to prevent loss of the flock to circulating avian influenza strains. Once a quality egg supply is secured, the eggs are inoculated with virus, the virus is incubated for a few days in the embryo, the allantoic fluid is harvested from the egg, and the virus is purified, inactivated, sterile filtered, and tested for potency. When all three strains have been produced, they are combined in a tri-valent vaccine, filled into vials, labeled, tested, released, and distributed to physicians throughout the world. Serious challenges have arisen when poor yielding strains greatly reduced the number of doses that could be made in a given production campaign. Likewise, the inactivation and purification process may be modified for a campaign to suit the particular vaccine strain. These challenges have proven to be too great for many

firms and the number of large-scale producers has dwindled in the last 5 years.

The final challenge to the task of providing flu vaccine to the customer is the filling process. This challenge will become even greater in the near future. Based on the logistics of when strains are selected (new strains require production of new test reagents to calibrate potency among manufacturers) and are ready for release, and the need for physicians to immunize before the flu season, manufacturing firms generally have just 15–17 weeks to fill sufficient vaccine to satisfy the demand for vaccine. Sterile filling and inspection of the product is often the rate-limiting step in the manufacturing process. The majority of influenza vaccine in many markets today is delivered in multi-dose vials. Increased demand for individual doses of product promises to put a tremendous strain on manufacturing capacity in the coming years.

For viral vaccines, replacing the embryonated egg or primary cell culture from animal organs has also been a challenge. For some viral vaccines, a mammalian cell line has been developed, optimized, and industrialized to produce a more consistent vaccine (e.g., poliovirus vaccines made on VERO cells). In the case of influenza, where the strains of virus change annually, the appropriate cell line for growth of multiple different viruses has been more elusive, although a number of new developments has brought promise of a new process in the next decade.

Inactivated poliovirus vaccine production was produced in primary mammalian cells from a new living animal for each lot of vaccine. To eliminate inconsistency, a cell line was developed from vervet monkey kidney and fully tested for safety (adventitious agents, etc.) and productivity (VERO cells). The VERO cell line was frozen in multiple aliquots for future production. The cell culture is grown from a frozen master seed and working seed was then produced in a quantity sufficient for future needs. Thawing of ampoules of working seed allow production of large quantities of cells (several thousand liters) in progressive stages. Once at production scale, these viable cells are infected with poliovirus (three unique strains are used) and the virus is harvested, purified, and inactivated. Similar to flu vaccine, three strains are combined in a final formulation and filled for distribution.

The use of cell lines for production has major benefits in process reliability, consistency, and robustness. Having a certified cell line for production eliminates the need to qualify new supplies of cells as must be done for influenza production in eggs, and also reduces the risks of extraneous agents and variability in the production process. VERO cells have the added benefit of growing in suspension, whereas many cell lines require attachment to a surface (micro-carrier) during growth, further complicating scale-up. Creation of a certified cell line involves tracing the history of the specific specimen used to create the master seed, and extensive testing for adventitious agents, as well as testing the ability to support viral propagation.

Recombinant vaccines have been successfully launched to prevent a number of bacterial (Lyme) and viral (Hepatitis B) diseases. This approach to manufacturing has many benefits over traditional approaches. Rather than growing the organism that is responsible for the disease, one can grow a harmless substrate into which the gene for a protein antigen that elicits a protective response in people has been inserted.

Choice of the host organism can be bacteria (*E. coli* for Lyme), yeast (Hepatitis B), or even a mammalian cell line. With proper choice of the host cell, tremendous manufacturing capabilities can be realized. Avoiding the direct handling of a pathogenic organism at large scale is a key benefit. One can also produce a modified protein through manipulation of the gene sequence (e.g., non-toxic pertussis toxin). Multiple gene copies can be inserted to further increase productivity. Using this technique, vaccines of very high purity have been developed. By avoiding the many contaminating proteins one might get from using the native organism, the capability of characterizing the product is improved. This enhances product licensing.

The challenges that recombinant vaccines present are the need to eliminate the host cell contaminants in the purification process as well as stability of the genome and the host cell itself in preparation of master seeds. The possession of intellectual property for the genes of protective antigens also presents a significant hurdle when implementing this approach.

An example of the steps in modern vaccine manufacture is given in Table 11, which concerns inactivated influenza vaccine.

Current problems

Three major problems for the future of vaccine manufacture are safety, cost, and adequacy of supply. A risk-adverse society demands from regulatory authorities and industry more guarantees of safety, however illusory. There is less and less tolerance of reactions, real or supposed. Legal liability in developed countries extorts heavier financial burdens, and they are just as heavy for false claims as for real ones.

There are multiple factors contributing to vaccine cost. Elaborate safety tests in animals and more elaborate observations in phase 3 trials are the two major ones, and it is estimated that the cost of development of a single vaccine varies between $300 million and $800 million. The manufacturer must then recoup its costs in developed countries before time and competition turn the vaccine into a commodity. Developing countries must then wait until market forces drive the price down or a donor decides to buy the vaccine for their use.

One cannot be against increased safety of vaccines, but the effort to achieve it results in a reduction of the number of vaccines available, as there is only a limited amount of funds for vaccine development. The public demand for safety encourages greater regulatory demands, which then aug-

Table 11. Steps in the manufacture of influenza vaccine

Choice of strain
Preparation of attenuated reassortant
Preparation of seed virus
Acquisition of million of SPF eggs
Incubation for 11 days
Inoculation with monovalent virus (regulated time, temperature, humidity)
Sterile harvest
Clarify
Add formalin (regulated time, temperature)
Ultracentrifuge
Sucrose gradient purification
Dilution
Add detergent
Pool three strains
Test sterility, inactivation, potency endotoxin, protein, stabilizer, pH, salt concentration, animal safety
Sterile filling of vials

ment manufacturing costs, but which governments are unwilling to pay. Reduced profits discourage private investors from providing capital, which in turn results in less production, ultimately compromising public health.

Vaccine supply is inadequate because of the high costs of vaccine production facilities and also because of the limited number of manufacturers. Only the appearance of new manufacturers in developing countries, or alliances between manufacturers in developed and developing countries can solve this problem, which could become disastrous in the event of a pandemic of a new infectious disease.

Conclusion

The development and production of vaccines is a difficult process. Manufacturers must make difficult choices of how to spend limited resources. The process of development itself is fraught with failure, for biological entities do not obey the same invariable rules as chemical entities. Moreover, the process is a "delicate fabric" [6, 7] that could easily tear in the near future if more attention and resources are not invested in this activity. Nevertheless, the many successes of vaccines encourage manufacturers to continue to develop new ones.

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How can the industrial world help to implement new vaccines against poverty-related diseases?

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Introduction

Communicable diseases are both a consequence and a cause of poverty. The most disadvantaged in society are subject to more social risk factors for contracting infections such as HIV, TB and malaria. By destroying human capital, these diseases reduce the potential for economic growth and fuel a vicious cycle of poverty at the individual, national and regional level. Contrary to common belief, however, the majority of diseases affecting the developing countries can be effectively treated using existing medicines. The Essential Drugs List [1] from the World Health Organization (WHO) contains safe, effective treatments for the infectious and chronic diseases which affect the vast majority of the world's population. The excessive mortality rate of many infectious diseases in developing countries is, therefore, mainly caused by limited access to drugs, unavailability of health services and failure to use prevention strategies [2].

The greatest successes in combating infectious diseases in tropical countries have all been vaccine-driven. Vaccination has resulted in the eradication of smallpox, the imminent eradication of polio, and a dramatic reduction in the prevalence of many other infectious diseases. Given the poor infrastructure and incomplete health-care systems in many developing countries, vaccines are, in fact, often the only pharmaceutical product that can make a substantial impact on public health.

There are, however, still a number of major infectious diseases for which an effective vaccine does not exist. The number of people affected by, infected with and dying from HIV/AIDS, TB and malaria continues to rise, and these diseases continue to evade immunisation efforts. In 2003, almost 3 million people died of AIDS, and there were about 5 million new HIV infections [3]. TB kills about 2 million people every year [4], whilst malaria kills more than one million people annually. New vaccines to combat these poverty-related diseases (PRD) are therefore urgently needed.

The political mandate

The genomic revolution of recent years has provided some tremendous advances in molecular biology and scientists of today are better equipped than ever to design effective vaccines. With recombinant technologies and production methods, vaccines can, in principle, be designed to be safer, more potent and even cheaper than traditional vaccines. Development of vaccines for many tropical diseases has nevertheless proven to be particularly challenging, as exemplified by HIV/AIDS and malaria. There are still major scientific hurdles to overcome before many communicable diseases can be efficiently tackled; this will need ample financial resources as well as leadership and determination among all the stakeholders.

The governments of G8 countries have on several occasions acknowledged the importance of increasing R&D investment in new vaccines to fight communicable diseases in the developing world. The Evian Communiqué of 2003 urged member governments to increase incentives, as well as to establish suitable regulatory capacity and support for ethical clinical trials. The Sea Island Communiqué issued in June 2004 specifically called for expanded involvement of both the private and public sectors in AIDS vaccine development. In addition, the World Bank as well as the US, French and UK governments have all been active in advocating increased spending and incentives. President Clinton announced in 1997 that finding an HIV vaccine within ten years was a national goal for the USA. In 2001, the US National Institute of Allergy and Infectious Diseases (NIAID) published a Global Health Research Plan for HIV/AIDS, Malaria, and Tuberculosis. The plan identified vaccine research as an important priority, and outlined the Institute's plans for fighting infectious diseases by building sustained research capability domestically and internationally and enhancing international partnerships. Within the European Union (EU), a strong commitment to combat PRD was provided in the "Programme for Action on HIV/AIDS, malaria and TB" [5], which promised a multi-sectorial approach to increase the affordability, accessibility and availability of new interventions for PRD. This massive support has resulted in renewed interest for vaccine research by the public sector, and vaccine development is now supported through specific programmes of the European Commission (EC), the U.S. government, the Australian government, and the WHO.

Public sector failure

The public sector harbours a significant pool of knowledge and experience in vaccinology, but resources are often uncoordinated and used ineffectively due to structural problems. Immunological research is a key discipline at all medical universities, and many countries have one or more specialised institutions that are dedicated to research in immunology, epidemiology or

communicable disease control. Most of these are fully or predominantly publicly funded, while others, including such prominent institutions as the Pasteur Institute in Paris, are non-profit private institutions that operate on similar principles as public research centres. With few exceptions, however, the public sector is characterised by national priorities and human resources that are too scattered to make a significant impact.

Many of the institutions were established and developed to improve and protect the public health situation in their home country. Their main objective was to address the national research and health agenda, and they did so successfully in most of the 20th century.

Increased human migration means that threats to public health are increasingly found outside national borders, and many institutions will need to revise their objectives towards achieving a global health agenda. At the same time, the increased complexity of the pharmaceutical development process means that public institutions can no longer provide the critical mass to undertake a vaccine development programme on their own. The ability of the public sector to develop new vaccines has consequently been slowly eroded during the last 2–3 decades.

Reinventing translational research

Europe has historically had a dominant role in vaccine research ever since the BCG vaccine was discovered at the Pasteur Institute in Lille in 1921. Europe has the largest vaccine production in the world, and constitutes the largest single vaccine market. Europe is also the main vaccine producer for developing countries, supplying nearly 70% of their vaccines. Europe should therefore have a strong scientific and moral impetus to spearhead the development of new vaccines for PRD.

From 1998 to 2002, the European Commission provided funding to a broad range of PRD research activities through the 5th Framework Programme for research (FP5). A total of 77 collaborative research projects were supported with 109 M Euro. More than 60 M Euro (corresponding to 55% of the total budget) was allocated specifically to vaccine research, confirming the renewed focus on this field in combating PRD.

A major achievement during the course of FP5 was the creation of a common "European Research Area" concept for interventions against PRD. Previously small, fragmented efforts by individual research teams became part of a dense network of numerous trans-national collaborations. The projects in FP5 thus involved more than 500 research groups, representing 21 European countries as well as 40 countries outside Europe.

The integration of European research continues in the 6th Framework Programme (FP6, 2002–2006), which also represents a substantial increase in public funding. Compared to FP5, the indicative budget for PRD in FP6 is nearly 4 times higher, amounting to a total of 400 M Euro, much of which

Table 1: Europe's current portfolio of large integrated projects for PRD vaccine development. The European Commission presently supports three large-scale research consortia on translational research of new vaccine candidates for PRD, namely MUCVAPRED (Mucosal Vaccines for Poverty Related Diseases), TBVAC (Tuberculosis Vaccine Development) and AVIP (AIDS Vaccine Integrated Project). Additional project consortia are planned for development of HIV/AIDS and malaria vaccine candidates.

	MUVAPRED	TBVAC	AVIP
Disease focus	HIV/AIDS, TB	TB	HIV/AIDS
Total budget	€ 18 M	€ 21 M	€ 15 M
EU contribution	€ 15 M	€ 17 M	€ 10 M
No of participating research groups	24	30	15

will go to research on vaccines. FP6 includes two new instruments that will be used to further integrate European vaccine research in PRD: the discovery and development of new vaccine candidates are supported through Integrated Projects (IP), whereas clinical trials of promising candidate vaccines will be supported through the European and Developing Countries Clinical Trials Partnership (EDCTP).

IPs are organised as large-scale collaborative projects, which should bring together a critical mass of partners, private as well as public, to undertake "translational" research. Project consortia must have the capacity to develop new vaccine candidates from the bench into products that can be tested on humans. Two years after the launch of FP6, 3 such vaccine consortia have been launched with financial support from the European Commission (Tab. 1) while another 2 consortia are planned. All consortia have established genuine partnerships between academic scientists, industrial vaccinologists and research teams from disease-endemic countries. The aim is to create synergy between academic expertise and the applied research of the pharmaceutical industry, thereby strengthening the translational vaccine research. The challenge ahead for European vaccine research will be to align the IPs into a single pipeline with a balanced and broad portfolio of new vaccine candidates, ranging from early discovery to clinical trials.

The EDCTP is an ambitious attempt to integrate the national programmes of 15 European countries in the area of clinical trials for PRD. It aims at establishing harmonised approaches and joint implementation of clinical activities among the participating countries. The EDCTP was established in autumn 2003, and has a target budget of 600 M Euro for its first 4 years of operation. The European Commission has allocated 200 M Euro to this initiative, while another 200 M Euro come from the 15 participating European countries. The remaining 200 M Euro will be sought from private sources, the R&D industry, foundations, charities and development funds. The EDCTP will fund phase II and III clinical trials from both the private and public sectors, including industry, non-profit organisations and individuals from Europe, developing countries and elsewhere. A further aim of the

EDCTP is to support capacity-building at clinical sites in Africa, thereby creating a long-term research partnership between developed and developing countries in the fight against PRD.

Market failure

The incompleteness of the public sector, as mentioned above, is aggravated by an evident research gap in PRD in the private sector. The routine use of vaccines saves millions of lives every year, but the economic value associated with them is negligible in comparison with the total pharmaceutical market. In most countries, vaccines are distributed by the public sector and once a pharmaceutical company has developed a new vaccine, governments will often use their powers as regulators and dominant purchasers to keep prices low. In spite of a very large sales volume, the world-wide annual turnover of vaccines therefore only constitutes approximately 2% of the total pharmaceutical market.

In today's pharmaceutical market, candidate products must have a high potential return-of-investment as well as a manageable risk profile. New development projects are therefore selected in strong inter- and intra-company competition. This will often eliminate vaccines, which generally have a low price level combined with relatively high manufacturing costs. Many pharmaceutical manufacturers have therefore replaced their vaccine research by drug development projects, and the number of vaccine companies in Europe and the US has steadily declined during the last 30 years. This situation is particularly pronounced for new vaccines aimed at PRD. During the last decade, vaccine shortage has thus become a serious problem affecting even developed countries.

Two recent studies concluded that the cost of developing a new pharmaceutical product is around US$ 800 million [6]. This figure includes the costs of failure and opportunity cost of capital in developed countries, but controversy surrounds the actual cost of developing a new vaccine for use in developing countries. The costs may be relatively low according to studies by International AIDS Vaccine Initiative (IAVI) and the global alliance for TB drug development [7], while others have argued that development of new vaccines could be difficult and costly if clinical testing were to be carried out locally in low-income countries with poor infrastructure and no, or insufficient, capacity for undertaking clinical trials.

In high-income countries, effective intellectual property protection enables inventors to recoup R&D investments by ensuring a limited period of exclusive marketing rights for a new product. The combination of a market with sufficient purchasing power and enforced intellectual property rights is therefore the basis for generating profit from private investments in pharmaceutical R&D. In this equation, vaccines for poor countries are obviously less profitable for the innovating company than would be drugs

destined for developed countries. Weak intellectual property protection in many parts of the developing world may further discourage private enterprises from focusing on PRD. The traditional business model for R&D investment is therefore challenged by the economic reality in developing countries, where a true domestic market for pharmaceutical products is either non-existent or at best unpredictable. Local financing for vaccines or medicines has been largely unavailable while external funding and donations have been random and unsustainable.

A number of non-economic factors may encourage a pharmaceutical company to pursue a PRD vaccine project. Most important would be the desire to demonstrate corporate social responsibility, but a new vaccine programme could also be part of a company's internal research capacity-building, or its strategy to expand its product range or geographical market. Private investment in PRD vaccine research has nevertheless remained negligible in recent years, and this situation is unlikely to change unless new motivating factors appear.

Accelerating clinical testing

The majority of R&D costs for new drugs and vaccines can be attributed to late-stage clinical trials. This is particularly true for new prophylactic vaccines that may have relatively low costs in the preclinical stages, but require very large clinical trials involving thousands of individuals. The cost of clinical development is also influenced by the dynamics of the target disease. For diseases such as HIV/AIDS and TB, several years of clinical trials in very large cohorts are necessary before a new vaccine can be released. Clinical trials of such an extensive nature consume huge resources and costs can be highly prohibitive for an otherwise cost-efficient R&D project.

A number of countries provide grants for clinical trials of new vaccines through their national research programmes. National institutions supporting clinical vaccine research include the US National Institute of Health (NIH), the French Agence Nationale de Recherche sur le SIDA (ANRS), and the U.K.'s Medical Research Council (MRC), but none of these initiatives have so far established a mechanism to provide sustainable support for full clinical programmes.

Some countries have further introduced special tax incentives to encourage private vaccine research in specific disease areas. In 2003, the UK government enacted the Vaccine Research Relief tax incentive for research and development related to AIDS, TB and malaria. In the US, tax credits are granted for costs derived from clinical testing of pharmaceuticals with orphan drug status, which would also include PRD vaccines.

Public-private partnerships (PPP) have recently become the most popular set-up for financial risk-sharing of R&D costs in the field of PRD (Tab. 2). Most of these ventures are established as separate legal entities

Table 2. Major product development public-private partnerships that are actively engaged in development of vaccines for PRD. Information about total funding originates mainly from the database of the Initiative for Public Private Partnerships for Health (www.ippph.org), while information about principal financial donors mainly originates from the websites of the respective PPPs. Public sector donors are indicated by country of origin or name of international organisation.

Disease focus	HIV/AIDS		Malaria		TB	HIV/AIDS, Malaria, TB
Public-Private Partnership (PPP)	International AIDS Vaccine Initiative (IAVI)	South African AIDS Vaccine Initiative (SAAVI)	European Malaria Vaccine Initiative (EMVI)	Malaria Vaccine Initiative (MVI)	AEREAS	European Developing Countries Clinical Trials Partnership (EDCTP)
Total funding	> 270 M US$	> 25 M US$	N/A	> 150 M US$	> 100 M US$	400 M Eur
Established	1996	1999	1998	1999	1997	2003
Major private charity donors	B&M Gates, Crusaid, Elton John, JM. Lloyd Mercury-Phoenix, MacArthur, Pendleton, Perls, Rockefeller, Sloan, Starr, Vanderbilt			B&M Gates, Rockefeller	B&M Gates	
Public sector donors	CA, DK, EU, GB, IE, NL, NO, SE, UNAIDS, US World Bank	EU, US, ZA	DK, EU, IE, NL, NO, SE		US	AT, BE, DE, DK, EU, ES, FR, GR, IE, IT, LU, NL, NO, PT, SE, UK
Pharma and healthcare industry	Becton, Dickinson & Company, GlaxoSmith Kline					
Other industry	Angel Music Ltd, DHL, MSN, Real-Networks, Yahoo, Viacom	Eskom, Implats, Transnet				

with relatively lean organisations, and with an objective to bridge the gap between preclinical research and early clinical trials. The PPPs involve collaboration between industry, academic institutes, and researchers in disease-endemic countries to develop new products for PRD. They operate by extensive outsourcing, which gives them the flexibility to quickly adapt to new circumstances, while their status as separate entities give them the freedom to attract funding from different sources. Roughly US$ 2 billion have been collected by drug and vaccine PPPs in the last 5 years, but additional financing required through 2007 is estimated to exceed US$ 1 billion [8].

Most of the vaccine PPPs focus their interest on a single disease, and the most mature of these ventures have built up sizeable portfolios of vaccine candidates that are entering the clinical testing stage. However, none of the PPPs have yet been able to bring a product to market, and the lack of success may be partly attributed to a limited involvement of industry. When the first PPPs were launched, it was generally expected that industry would contribute with active participation and direct funding, but this has only happened to a limited extent.

The vast majority of funding has so far come from philanthropic organisations such as the Bill and Melinda Gates Foundation or the Rockefeller Foundation, which is a pioneer in this area. European governments and the USA are the second largest group of donors, while multilateral donors such as the EU, UN and the World Bank have provided relatively small amounts. Industry have mainly contributed with "in-kind" donations such as staff, technical know-how and access to testing facilities.

Compared to the disease-specific PPPs mentioned above, the EDCTP initiative has a broader scope, as it addresses all of the three major PRDs. The EDCTP is unique in having started as a purely publicly funded initiative (PFI), although the active participation of private stakeholders is encouraged. The EDCTP is also unique in focusing on late-stage clinical trials, where the need for both financial and structural support becomes evident. An important aspect of the EDCTP is therefore to support capacity-building at clinical sites in Africa, thereby creating a research partnership between developed and developing countries that can accelerate the clinical testing of new disease interventions.

Given adequate coordination, the disease-specific PPPs and the EDCTP may effectively interact to support the full clinical development of new vaccine candidates (Fig. 1). With sufficient financial backing, PPPs could fill a crucial gap in the development pipeline by moving promising new vaccines from preclinical through early clinical phases. The EDCTP may then provide support through late-stage clinical trials and up to the regulatory phase.

Jumping the regulatory hurdles

Before a new pharmaceutical product can reach the market, it needs to be

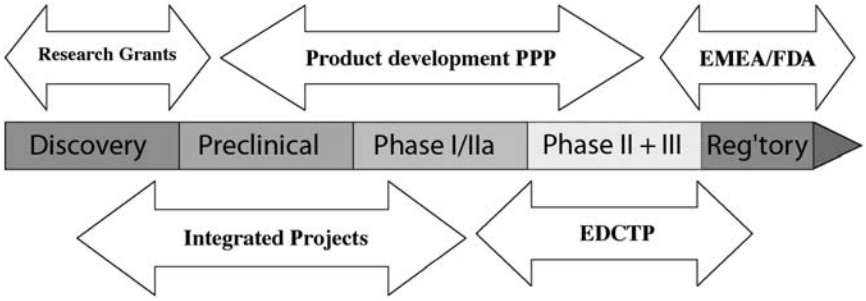

Figure 1. Simplified overview of the different steps for developing a new vaccine, and indication where existing support mechanisms have their primary intervention.

examined and approved by the relevant regulatory authorities. Provisions to facilitate the regulatory process exist in many countries for rare or orphan diseases. In 1983, the US Congress introduced the Orphan Drug Act for diseases affecting fewer than 200,000 persons in the United States. Vaccines for PRD would therefore technically fulfil the US orphan definition. Incentives include a 50% deduction tax credit for clinical trial expenses, formal protocol assistance and a market exclusivity of seven years. More than 200 orphan products have already been brought to market under the Orphan Drug Act [9]. As of 2000, however, only 8 candidate vaccines had been designated to have orphan status, and none of these had reached the market. The same is true for the EU, where an orphan drug regulation was introduced in 2000 with 10 years market exclusivity as the most important element. The US and EU experiences show that existing orphan regulations fail to stimulate vaccine development for PRD, mainly because market exclusivity in the US and/or EU is virtually irrelevant for PRD products. Vaccines for developing countries could thus be defined as "economically orphan" due to small markets rather than prevalence or burden of disease, and special incentives should be considered to facilitate their regulatory approval.

Using existing flexibility to reduce regulatory barriers to innovation is of current interest for the European Medicines Agency (EMEA), the US Food and Drug Administration (FDA) and a number of national authorities, and this possibility should be fully exploited to push the approval of new vaccines. A specific regulation for economically orphan products could introduce a number of additional facilities, including fast-track procedures, waiving of registration fees, scientific advice to develop medicines for resource-poor settings, and advice leading to Good Manufacturing Practice (GMP) and Good Clinical Practice (GCP) certification. Regulatory agen-

cies could also assist with protocols for clinical trials and compilation of the regulatory dossier from an early stage. This may further be combined with special provisions for granting extended marketing exclusivity beyond the expiry of standard intellectual property rights.

The regulatory process in developing countries should also be facilitated. An important step could be to replace the individual national approvals of new products in developing countries with regional procedures of a type similar to those of EMEA in Europe. The recent revision of the EU pharmaceutical legislation enabled the EMEA to give a scientific opinion for the evaluation of medicines to be marketed outside the EU [10]. This procedure allows EMEA to review new products intended exclusively for use in developing countries. This could be the first step towards establishing regional approval procedures in parts of the world other than Europe, with support and guidance from EMEA.

New incentives to encourage R&D investments

Reduced regulatory requirements can surely save development costs and speed up approvals, but additional and innovative incentives will be necessary to boost private investments in PRD vaccines (Tab. 3). Incentives to encourage the development of new products can be broadly divided into push mechanisms, which finance research input, or pull mechanisms, which reward innovators for actually bringing a new product to the market. As summarised above, several ambitious push mechanisms have been established in recent years to predominantly support vaccine research within the public sector, but the vaccine manufacturers have often stated that the most attractive incentives for them are pull mechanisms.

Purchase funds

The optimal pull mechanism would be the creation of a stable, sustainable market for vaccines in the least developed countries. This cannot be realised in the short or medium term unless the developed countries finance it through purchase mechanisms established by public organisations or private charities. The Vaccine Fund under the Global Alliance for Vaccines & Immunization (GAVI) and The Global Fund to fight AIDS, TB and malaria (GFATM) are reflections of this idea, but the historic underfunding of purchase funds, the unpredictability of support from developed countries and the tendency to purchase existing rather than future products has so far made this option ineffective as an R&D incentive. To act with full impact, funding would have to be dedicated to new products rather than existing ones, and an appropriate legal framework as well as a credible and long-term funding mechanism would have to be secured. One

way to structure a scheme for advance purchase of vaccines would be that potential sponsors such as governments, international organisations or private charity foundations make a legally binding commitment to pay a guaranteed price for a specified number of doses of a new vaccine with defined characteristics. If a commitment failed to produce an effective vaccine, no donor funds would be spent, making this a potentially cost-effective mechanism to stimulate R&D on vaccines. Given the complexity of vaccine development, purchase funds would nevertheless have to be very large to serve as a real incentive for private R&D investments. A recent study by Kremer and Glennerster [11] concluded that an advance purchase system could work with a guaranteed price of US$ 15–20 per treatment for the first 200 million individuals vaccinated, and the rest of the vaccine supply being priced at a modest mark-up over manufacturing cost. In the foreseeable future, it appears unlikely that potential sponsors will be prepared to make firm, legally binding promises to such financial commitments that amount to more than US$ 3 bn. Even if it happens, the individual vaccine developer will have to be convinced that funders or their successors will not try to avoid such commitments when they arise, given the huge rewards at stake and any potentially conflicting interests between sponsors and vaccine companies.

Transferable exclusivity rights

The possibility of transferring exclusivity or special privileges from one product to another can potentially be a very powerful incentive. Transferable market exclusivity covers the principle of granting extended market exclusivity for a pharmaceutical product in Europe and/or the US, as a reward for developing a new (but entirely different) vaccine for treatment of PRD. In exchange for such exclusive benefits, it would be reasonable to require that the company should also commit to produce and sell the PRD vaccine at a low price in developing countries. The prospect of extending the market exclusivity of a highly profitable, yet unrelated pharmaceutical product could create a strong incentive for vaccine development, but it may stimulate R&D at a high and unevenly distributed cost. The annual sales of a pharmaceutical blockbuster product is around 250 million Euro in Europe (more in the US), and the buyers of such pharmaceutical products would carry the entire burden of financing new vaccines for PRD. Most health-care expenditure in Europe is funded through public health insurance or welfare schemes, but health-care budget deficits are widening in many countries and extensions of marketing exclusivity will therefore be seen as an unwelcome increase in drug-related costs.

To make the incentive attractive to smaller companies without "blockbuster products" that could receive the extended exclusivity rights, such rights would have to be tradable between companies (e.g., though auction-

ing). An additional problem relates to the impact of new vaccines and how to evaluate them. Should all new vaccines elicit the same exclusivity extension or should the length of extension be related to the efficacy, safety, manufacturing costs, etc.? If a special committee or agency is supposed to rule on such matters, it will be an essential but difficult task to establish its credibility and independence. Given the large financial rewards at stake, the committee should be detached from stakeholders in both the private and public sector.

A more manageable and less controversial system could be envisaged by limiting the transferable privileges to a fast-track regulatory procedure. In such a scheme, development of a PRD vaccine would elicit a right to process another, unrelated product through a fast-track regulatory approval. The other product, which could be a potential blockbuster, may thus reach the market earlier than expected. This would enable a company to generate profit sooner and for a longer period. In a highly competitive situation, it could also equip the innovating company with a "first-on-the-market" advantage. If the gain in market time is sufficiently high, such a scheme could be attractive as an incentive and at the same time manageable through the existing regulatory procedures. Fast-track provisions already exist in both the US and EU pharmaceutical legislation, and flexible use of these mechanisms could allow the introduction of a transferable fast-track privilege system without the need to pass new legislation.

Cash premiums

Cash premiums can be seen as a simple mechanism to reward research results in PRD vaccinology, either on its own or in combination with other incentives. Once a new vaccine has reached the market, the cash premium would be awarded to the innovating company. The risk of development would be borne entirely by the industry, but a predefined reward would be paid out in case of success. On a stand-alone basis, the premium for developing a new vaccine would have to be substantial and probably be at least equal to the average development costs of a new vaccine. The actual size of the premium could also be made dependent on the therapeutic potential of the new product. In its most elaborate version, cash premiums thus share many similarities with advance purchase commitments, including many of the same weaknesses. However, cash premiums could also be set up in a simplified version as an international award that would elicit a substantial financial reward to the innovating company as well as prestige and world-wide recognition to the scientists involved. The financing of such an award can be envisaged through different mechanisms, either directly by the public sector through tax revenues, by private sponsors or charity foundations. Part of the reward money could also be accumulated from regula-

Table 3. A comprehensive summary of existing (marked with *) and proposed incentives. Incentives are grouped according to their phase of intervention in relation to the vaccine development pipeline

Basic research & discovery - Publicly funded R&D* – e.g., core research at public universities and research institutions - Research grants* – financial support to specific research projects from public, private or charity sources.
Early development/translational research - Large-scale collaborative projects* – e.g., EC-supported integrated projects - Public-private partnerships* – e.g., disease-specific PPPs with focus on translational research - Offsetting taxation against R&D expenditure* – e.g., UK regulation
Clinical development - Publicly funded initiatives for late stage clinical trial* – e.g., EDCTP - Government indemnification of damages caused by side-effects (e.g., for vaccines) - Clinical trial protocol support – simplified clinical requirements - Low-cost loans for clinical trials and establishment of manufacturing facilities
Regulatory - Existing orphan drug procedures* – e.g., the orphan drug regulations of FDA and EMEA - Special regulatory processing for economically orphan products – e.g., including fast-track procedures, waiving of registration fees, scientific advice to develop medicines for resource-poor settings, and advice leading to GMP/GCP certification. - Regulatory review by EMEA of products for use in developing countries*
Market related - Increased market exclusivity for economically orphan products – e.g., as in the orphan drug provisions - International IPR extensions – longer IP for relevant new products - Transferable market exclusivity as reward for developing a new PRD vaccine - Transferable fast track as reward for developing a new PRD vaccine - Advance commitments to purchase new vaccines - Cash premiums - International Awards – worldwide recognition - Tax credits for sales or donations to developing countries* – e.g., the US regulation

tory fees paid by the pharmaceutical industry for approval of pharmaceutical products for the developed part of the world. Interestingly, this type of risk-reward model seems to be working for another high-risk, high-cost programme, namely the commercial space travel enterprise (the X-Prize foundation will reward a cash prize of 10 M USD to the first team that privately finance, build and launch a spaceship that can carry 3 people into space.).

A global research policy for PRD is needed

In recent years a substantial increase in resources has become available to combat PRD in developing countries. This is most noticeable in the case of

Table 4. The average annual EC financial contribution to fight HIV/AIDS, malaria and tuberculosis in developing countries in the period 2003–2006 compared to 1994–2002 (in million Euro). Total and research contributions are indicated.

	HIV/AIDS		Malaria		TB		Total		Annual average	
	1994–2002	2003–2006	1994–2002	2003–2006	1994–2002	2003–2006	1994–2002	2003–2006	1994–2002	2003–2006
R&D	93,1	200,0	55,4	100,0	59,2	100,0	207,7	400,0	23,1	100,0
Total	494,2	674,5	159,4	264,0	77,2	216,5	730,8	1178,0	81,2	294,5

HIV/AIDS, where the official development assistance to fight HIV/AIDS increased by 15-fold – from US$300 million in 1996 to almost US$5 billion in 2003. The intensified fight against PRD has been accompanied by an even steeper mobilisation of public and private funds for research projects, including vaccine-oriented development. Within the European Union, the EC has increased its contribution to PRD research almost 5-fold since 2002, while the overall contribution to combat PRD increased less than 4-fold (Tab. 4). The improved funding for vaccine research has also been accompanied by improved research structures for preclinical vaccine development, as exemplified by the large-scale IPs that have been formed across Europe.

It is noteworthy that large financial contributions to vaccine research in the public sector is increasingly coming from private non-profit organisations such as the Wellcome Trust, the Rockefeller Foundation and in particular the Bill and Melinda Gates Foundation, which has donated over US$1 billion to research, development and distribution of vaccines since it was established in 2000. The global increase in funding for PRD has made it possible to launch a number of new initiatives that can support vaccine development. Each of these initiatives may play an important role by filling different niches of the vaccine development process. This could provide an important momentum to the translational research, which is necessary to move any new vaccine candidates from discovery to early stages of clinical testing. The challenge ahead will be to integrate the many initiatives correctly, so they supplement each other rather than duplicate or compete for the same resources. A global approach is therefore needed, and stakeholders at all levels, industry and the public sector, developed and developing countries must coordinate their efforts. Global health should be made a national priority in developed countries and development of new vaccines should be supported according to their global public health values. Developing countries, for their part, should engage more actively in the R&D process as part of their national research and health agendas. Governments in developing countries must acknowledge the value of research as a long-term solution to public health problems. Developed and

developing countries should work together on lowering regulatory entry barriers for new vaccines.

The failures of both the private and public sector must be remedied to stimulate research in new vaccines against PRD. This will require sufficient funding, but will also require the creation of a suitable international framework, where private and public stakeholders are mutually motivated to develop new vaccines. In the area of HIV/AIDS, the recently launched Global HIV Vaccine Enterprise (GVE) is attempting to catalyse this process. The GVE is a virtual consortium to accelerate HIV vaccine development by enhancing coordination, information sharing, and collaboration globally. The concept was originally proposed by an international group of scientists [12] and took inspiration from the successful alliance and strategic plan that characterized the human genome project. The GVE was endorsed by G-8 governments at the Sea Island summit in June 2004, and was asked to develop a strategic plan for coordination of global vaccine development efforts and alignment of existing and new resources for this purpose.

On the political level, national policy-makers and health authorities in both developed and developing countries should therefore increasingly consolidate and coordinate their research priorities towards the global health needs. In this context, federal or transnational institutions like the WHO, European Commission, NIH and Japan's National Institute of Infectious Diseases (NIID) can play key roles by setting priorities, deliver organisational leadership and distribute funding. At the same time, new and more powerful incentives will have to be introduced to stimulate private investment in PRD vaccine research. Predictability and long-term commitment from the public sector in rich as well as poor countries are necessary to translate PRD research into new vaccines with societal value for disease-endemic countries. With sufficient funding and commitment from all stakeholders, the existing roadblocks for developing new effective vaccines can eventually be removed.

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Public-private partnerships

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New approaches towards development, production and use of developing-country market vaccines in developing countries

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Background

Inequity in access to existing and newly licensed vaccines has increased over the past two decades as new vaccines have become available at prices that most low-income countries are unable to afford. From the time of introduction of a new vaccine in Europe or the US, it usually takes a decade or more for the adoption of these vaccines in developing countries. For example, *Haemophilus influenzae* type b (Hib) conjugate vaccine has been used routinely in North America, most European countries and some Latin American countries for more than a decade with dramatic reduction of disease incidence [1–3]. Although this vaccine has also been demonstrated to be highly effective in preventing disease in developing countries, with few exceptions vaccine uptake in these countries has been slow [4]. Several factors have contributed to the slow introduction of these vaccines in the developing world. In many countries, policymakers may still have uncertainties about whether an adequate case can be made for introducing the vaccine into real-life public health programs in their countries. This is because they either have insufficient data on the local disease burden, the evidence provided by pre-licensure evaluations fails to address many of the practical questions about implementing a new vaccine in real-life programs, or they lack the capacity and infrastructure to deliver these vaccines [5]. However, the principal obstacle to introducing new vaccines has been their cost [6]. When Hib conjugate vaccines were first marketed, their prices were affordable only in industrialized countries. As manufacturing production capacity increased and competing products entered the marketplace, prices gradually declined. Eventually, prices became tiered, with sales of vaccine in developing countries set at a lower price than those in developed countries [4, 7, 8]. However, even with tiered pricing, these vaccines were still unaffordable for the majority of the poorest countries in the world [4] . The

failure to introduce these vaccines in countries with a GDP/*capita* below $1,000 is particularly notable. More recently, a 7-valent pneumococcal conjugate vaccine was licensed in the United States [9]. However, this vaccine is selling at a price of over US$50 per dose, which obviously restricts its use to affluent countries. Hib and pneumococcal diseases were important causes of disease in industrialized as well as developing countries. The pharmaceutical industry, therefore, was highly motivated to invest large sums of money in the development and licensure of these vaccines because of the high return on investment they could expect from sales of a high-priced vaccine in industrialized countries.

The recent availability of increased funding from international donors such as the Bill and Melinda Gates Foundation and the inception of the Global Alliance for Vaccines and Immunization (GAVI) and the Vaccine Fund [10] have opened new opportunities for the expanded use of Hib vaccines and the accelerated introduction of pneumococcal vaccines in developing countries. However, it is yet to be seen whether these funds will be enough to substantially accelerate the introduction of these vaccines, increase the limited capacity that currently exists for some of these vaccines in established vaccine manufacturers, and ensure their sustainable use in the poorest countries where the disease burden is highest [11].

In contrast with the examples above, there are a number of diseases that affect almost exclusively developing-country populations and pose little if any risk for individuals in industrialized countries unless they travel to disease-endemic areas. Historically, vaccines developed against these diseases have been termed "orphan" or "developing-market" vaccines [12, 13]. In the remainder of this paper the term "developing-market" vaccines will be used.

In general, the private sector has little financial incentive to produce developing-market vaccines since the returns on the investment needed for research and development, manufacturing capacity, conducting clinical trials and other activities needed for vaccine licensure are low, and these projects almost certainly compete with other high-value projects.

Developing-market vaccines can broadly be divided in two major groups. The first group consists of those vaccines against diseases prevalent in the developing world for which major R&D gaps still exist. Vaccines against dengue fever, malaria and TB belong to this group. For each of these diseases, science is a limiting factor for the development of effective vaccines. A combination of dearth of information about the pathogenesis of these diseases, technical hurdles, complex intellectual property rights and unattractive market potential has long made vaccine manufacturers very reluctant to invest large sums in R&D against these diseases. Only recently there appears to be glimpses of hope. The Bill and Melinda Gates Foundation has launched new programs focusing on the development and testing of new vaccine candidates against dengue, TB and malaria, through the Pediatric Dengue Vaccine Initiative at the International Vaccine

Institute, the Aeras Global Tuberculosis Foundation and the Malaria Vaccine Initiative at the Program for Appropriate Technology for Health (PATH) [14, 15]. These programs operate primarily through partnerships with academia, government, and biotech and pharmaceutical companies and aim at overcoming technical and financial hurdles to the development and clinical testing of promising vaccine candidates. They are also addressing potential barriers to eventual dengue, malaria and TB vaccine introduction, including market-related, intellectual property, and financing issues. While some programmatic, economic or ethical issues may be extrapolated from one disease to another, most of the scientific and technical barriers are disease-specific, and therefore would require a single chapter devoted to each of them.

There is a second group of developing-market vaccines that comprises diseases that constitute a major public health problem in developing countries, for which the technology for the development of effective vaccines already exists, but that are not developed by large vaccine manufacturers due to the lack of market opportunities. Bacterial enteric diseases (typhoid and paratyphoid fever, shigellosis, pathogenic *E. coli* and cholera), which kill around 2 million children per year, belong to this group. Even for vaccines that are of interest to populations in industrialized and less-developed countries alike, such as Hib, meningococcal or pneumococcal diseases, the situation is further complicated by the fact that for these vaccines, the industrialized and developing-country vaccine markets are diverging and differentiating. Factors including epidemiological variations by geographic region (circulating serotypes, carriage, co-infections), differences in cost-benefit ratios, characteristics of preferred formulations because of production and cold chain capacity may result in the development of vaccines that although suitable for industrialized countries might not be the most appropriate for developing-country populations. For example, certain formulations of pneumococcal conjugate vaccines may cover only serotypes prevalent in industrialized countries [16] and monodose thiomersal-free meningococcal vaccines may not be the most appropriate for mass immunization campaigns in the African meningitis belt [17].

For all of these pathogens, surface polysaccharides, in the form of capsule or lipopolysaccharide, are both essential virulence factors and protective antigens. Serum antibody to the surface polysaccharide confers protection against disease by activating complement-mediated bacteriolysis and/or opsonization. Parenteral polysaccharide vaccines against these diseases are safe and elicit protective serum IgG capsular antibody responses in adults or children older than 2 years of age, but are generally not immunogenic in younger children and infants. Because purified capsular polysaccharide acts like a T-lymphocyte-independent antigen, the serum antibody response cannot be readily boosted by administration of additional doses of the polysaccharide and do not induce immunological memory [18–22].

Glycoconjugate vaccines obtained by coupling these pathogens' polysaccharides with a carrier protein can overcome the limitations of existing polysaccharide vaccines against this group of developing-market diseases. However, Big Pharma is not likely to make a large investment in these vaccines for markets that are very unattractive. Thus, in order to guarantee a sustainable supply of these vaccines at affordable prices to programs for the poor in developing countries, alternative models for the development and large-scale production of conjugate vaccines need to be envisaged.

The remainder of this chapter aims at 1) demonstrating that conjugate vaccines are an essential platform technology for developing-market vaccines; 2) establishing the rational basis for the production of conjugate vaccines by a number of qualified local producers; 3) discussing the different models for transferring this technology to local producers for the accelerated introduction into and use of these vaccines by public health programs of developing countries.

Polysaccharide-protein conjugate vaccines are an important platform technology for developing-market vaccines against enteric and encapsulated respiratory bacteria

Polysaccharide-protein conjugate vaccines elicit immunological responses in infants and children

The development of polysaccharide-conjugate technology has been one of the most important developments in vaccinology and has allowed the production of several important vaccines such as those against Hib, meningococcal and pneumococcal diseases [23–25]. These vaccines are based on the observation that the immunogenicity of polysaccharides can be improved through chemical conjugation to a protein carrier, thereby eliciting a T-cell-dependent anti-saccharide antibody response [26, 27]. The resulting polysaccharide-protein conjugate vaccines are safe, immunogenic in young infants and induce long-term protection. For some pathogens, immunization with conjugate vaccines also decreases nasopharyngeal carriage and transmission of the organism. Vaccines with these properties are suitable for introduction into the Expanded Program on Immunization (EPI)

The public health impact of conjugate vaccines has been impressive. Vaccination with Hib conjugate vaccines has nearly eliminated Hib disease in affluent and middle-income countries [1–4]. In addition, a polysaccharide-protein conjugate vaccine against *S. pneumoniae*, recently licensed in the U.S. and Europe, was highly effective for the prevention of pneumococcal bacteremia and meningitis in clinical trials [9]. Widespread use of this vaccine is expected to eliminate invasive disease caused by the seven strains contained in the current vaccine (responsible for the majority of cases in infants and children). Finally, a group C meningococcal con-

jugate vaccine is part of the routine infant vaccination program in the UK and has resulted in a marked reduction in meningococcal disease [28]. Other countries, including Spain, Germany and the Republic of Ireland, have also introduced this vaccine into their routine infant immunization programs.

Polysaccharide-protein conjugate vaccines against enteric and respiratory bacteria have the potential to prevent at least three million deaths a year in the developing world

Hib and pneumococcal conjugate vaccines can prevent at least half of pneumonia cases. In the future, conjugate vaccines could virtually eliminate bacterial meningitis. Besides conjugate vaccines against Hib, pneumococcus and group C meningococcus, vaccine manufacturers are currently developing conjugate vaccine combinations incorporating groups A, C, Y and W-135 meningococcal polysaccharides [24]. In addition, a N-propionylated polysialic acid from *Escherichia coli* K1 polysaccharide capsule coupled to purified recombinant PorB outer membrane protein as a carrier is being tested in humans against group B meningococcus [29]. Finally, prototypes against group B streptococci [30] have also been developed and clinically evaluated. For all these vaccines, a variety of formulations and presentations not currently considered by large vaccine manufacturers either as stand-alone products or in combination with other antigens, may be required to accommodate the needs of individual countries or regions in the developing world

Furthermore, conjugate vaccines could also potentially drastically reduce mortality caused by enteric bacteria, such as *S. typhi* and *S. paratyphi*, *Shigellae*, *E. coli*, *V. cholerae O1* and *O139*. Conjugate prototype vaccines against typhoid fever, paratyphoid fever, *E. coli* O18 and shigellosis [31–34] have been shown to be safe and effective in proof-of-principle trials. Furthermore, prototypes against *V. cholerae* spp have been developed in the laboratory [35, 36] (Tab. 1).

Big Pharma will not likely be a source of most polysaccharide-protein conjugate vaccines for developing-country populations

Big Pharma is unlikely to give priority to developing a new generation of developing-market conjugate vaccines because it will not be able to obtain an adequate return on investment. Public sector attempts to induce industry to produce these vaccines by offering to offset the direct costs are unlikely to succeed because of the opportunity costs involved.

The public sector has attempted to influence the decisions of the research-based industry to develop new vaccines for use in the poorest

Table 1. Estimated deaths caused by pathogens against which conjugate vaccines are or could be developed

Pathogen	Estimated deaths/year
S. pneumoniae	1,000,000 [37]
H. influenzae type b	300,000–500,000 [38]
N. meningitides	30,000–60,000 [39]
Group B streptococci	Unknown
Non-typeable *H. influenzae*	Unknown
Shigella	1,100,000 [40]
Enterotoxigenic *E.coli*	380,000 [41]
V. cholerae	120,000 [42]
S. typhi and *S. paratyphi*	600,000 [43]

countries by a variety of means. Overall the results have been disappointing. Appealing to corporate altruism or provision of incentives ("push strategies") such as supporting the cost of clinical trials, strengthening of field sites in developing countries, R&D tax credits and small business grants, fast-track regulatory reviews, standardizing serological assays, and developing international recommendations for quality control and production, while useful in themselves, are rarely sufficient to influence the decisions of major manufacturers.

Notwithstanding the new funding opportunities that have emerged recently, public sector alliances with established manufacturers for the development of vaccines that largely target developing-country populations have proven to be difficult. Group A meningococcal conjugate vaccine is a revealing example [17].

In May 2001, the Bill and Melinda Gates Foundation awarded a US$70 million, 10-year grant to WHO and PATH to support the Meningitis Vaccine Project (MVP), with the goal of eliminating meningococcal epidemics in Sub-Saharan Africa. Meetings were held with major vaccine manufacturers to discuss ways to stimulate commercial development of a low-priced group A meningococcal conjugate vaccine. Possible incentives included providing capital investment in the form of a low-interest loan for increasing manufacturing plant capacity; underwriting costs of process development, production of investigational vaccine lots and conduct of clinical trials; and forming a partnership for joint management of clinical, serology and regulatory activities. Although several vaccine manufacturers in the US and Europe with expertise in conjugate or meningococcal vaccines were approached, only two companies made formal proposals. The most important obstacle for all of the companies was the perceived opportunity costs of the project. Their business models were based on the development of innovative products with high potential returns on investment. Developing a low-cost group A meningococcal conjugate vaccine for the meningitis-belt countries in Africa, and expanding manufacturing capacity, were major undertakings that would directly compete with resources need-

ed for other higher-value projects. Some of the manufacturers indicated that the opportunity costs of the project would likely result in loss of revenue, even if all the costs of development of the group A meningococcal conjugate vaccine were subsidized [17].

More critical than subsidizing the costs of vaccine development or the capital investment required for building manufacturing capacity was the assurance of a profitable future revenue stream that would represent a sufficient return on investment to offset the high opportunity costs of the project, such as the guaranteed purchase of a large number of doses at a profitable price: the so-called "pull mechanism". This purchase-precommitment approach has as its most attractive feature the fact that it only rewards successful outputs rather than supporting research that may not succeed [44]. However, the public sector has yet to develop effective mechanisms by which to guarantee long-term vaccine purchases, particularly for a vaccine that was not expected to be available for the next several years.

One may argue that the MVP focused on the poorest countries in the world where the possibility of tiered pricing is very limited. For other vaccines, the existence of middle-income country markets may somehow prove a greater incentive to get vaccine manufacturers involved. This is only partially true. Whereas, theoretically, countries in Asia and Latin America provide greater possibilities for profit for vaccine manufacturers, increased costs for R&D and stringent regulatory requirements have pushed vaccine corporations to look increasingly for "blockbuster" products with high potential returns on investment. Furthermore, even for vaccines against group A meningococcus, one could have argued that potential markets existed for Middle Eastern and several Asian countries, but these prospects clearly did not lure manufacturers to pursue the development of the vaccine. It is unrealistic to expect that for other vaccines the same reasoning would not apply.

The role of local producers in the production of conjugate vaccines against developing-market diseases

Background

In the last few years, a new group of qualified local producers has emerged in the developing world. This group has not only greatly improved their ability to produce high-quality vaccines, but has also established robust financial systems and a managerial structure with clear lines of responsibility, accountability and authority. Indeed, two-thirds of the traditional EPI vaccines supplied to UNICEF come from manufacturers located in the developing world (Tab. 2) that have been previously pre-qualified by WHO [45].

Table 2. Distribution by source of vaccines purchased through UNICEF tenders [45]

	UNICEF supply specific to Global Fund purchase (Hep B, Hib, DTP-HB, DTP-HB-Hib)	UNICEF supply for basic EPI vaccines excluding OPV
Established vaccine manufacturers	73%	36%
Developing-country vaccine manufacturers	27%	64%

The pre-qualification system [46] depends on effective regulatory oversight. For vaccines, regulatory oversight includes adequate review of preclinical and clinical data for licensing, including review of the product file and assurance that the proposed facility is constructed and run in accordance with principles of Good Manufacturing Practices (GMP). In addition, it includes activities carried out post-licensing: lot release, regular GMP inspections, monitoring for field impact (including investigation of adverse event reports), resolution of complaints, and review and approval of changes in the license.

Manufacturers in the developing world producing vaccines pre-qualified by WHO include the Serum Institute of India and Shantha Biotechnics, India; Biofarma, Indonesia; Biomanguinhos, Brazil; CIGB, Cuba; and the Pasteur Institute, Senegal.

Another group of manufacturers with the necessary regulatory environment for safety and quality that are not yet pre-qualified include Panacea Biotech, Biological E and Bharat Biotech International, India; Vabiotech, Vietnam; Instituto Finlay, Cuba; and Instituto Butantan, Brazil. Finally, a third group of manufacturers that have made demonstrable steps to upgrade their oversight for quality and safety include Birmex, Mexico; Vacsera, Egypt; the Pasteur Institute, Iran; and the Lanzhou Institute, Chengdu Institute, and Shenzen Kangtai, China.

Several of these institutions have already shown the ability to acquire new technologies to develop a new generation of vaccines.

Unfortunately, the market for more profitable vaccines, such as Hep B, Hib conjugates and especially their combinations, is still dominated by established vaccine manufacturers (Tab. 2). In the last few years, though, the situation has been gradually changing with more manufacturers producing high-quality Hep B vaccines. However, the ability of local producers in the developing world to produce conjugate vaccines is still very limited. Only a sustained program of technology transfer, technical assistance and training will speed up this process.

Big Pharma is increasingly focusing its R&D efforts on products aimed at lucrative markets. Thus, for vaccines against diseases affecting populations in the developing world, the possibility of establishing partnerships between the public sector and qualified local producers is becoming

increasingly vital; for conjugate vaccines, these alliances are becoming even more important. From an economic point of view, such alliances make sense. What Big Pharma normally views as an opportunity cost, local manufacturers may view as a good opportunity for: 1) potential capital investment for vaccine production and up-grading of their facilities; 2) increased sales of vaccines at a low price according to Western standards but at a profitable price for them; and 3) acquisition of conjugate technology/know-how for the development of a broad range of products that could, in the future, be sold in more profitable markets.

Conjugate vaccines can be produced locally but the process is demanding

As a consequence, transfer of technology will only be practical for a small group of the best WHO qualified manufacturers. The manufacturing of conjugate vaccines has 4 elements:

Availability of a purified carrier protein

Among the different carrier proteins that can be used for conjugate vaccines, tetanus toxoid (TT) stands out as the most appropriate for local production of conjugates for two important reasons: cost and availability. The cost of TT, based on international quotations for bulk TT, is negligible. Using TT of good purity at 1,500 Lf/mgPN (WHO specification ≥ 1,000 Lf/mgPN [47], one dose of vaccine (20 μg protein) translates to about 5 Lf of TT, or assuming 50% yield, 10 Lf of TT consumption. The corresponding cost is between US$0.01–0.02/dose. With regard to availability, several institutes and manufacturers in the developed and developing world have TT in production, or have established an agreement with a manufacturer to acquire it.

Availability of purified polysaccharide

The preparation of the polysaccharide (PS) involves fermentation of the bacterial strain, extraction of the cell wall and purification. Guidelines for glycoconjugate vaccines require that the PS meet agreed specifications for PS, residual protein, nucleic acids and endotoxins [48]. Local producers can acquire it from contract manufacturers such as Synco Ltd [17], or they can establish the methods in house, which is the better long-term option. The latter will require the preparation of master and working cell banks, optimization of fermentation processes and setting up the purification technology in a GMP facility.

Availability of a conjugation facility

Conjugation requires a small dedicated facility consisting of a room equipped with an exhaust hood for activation chemistry, a chromatographic unit for conjugate purification, and a recipient class A area for sterile filtration of the conjugate. All these elements would be grouped together. The chemical processing room (6–8 sqm) and the chemicals warehouse have special requirements, as the process uses toxic and volatile chemicals.

Acquisition of conjugation technology

Several technologies that are used for the production of existing Hib conjugate vaccines may be considered by local producers for production of other conjugate vaccines.

The PRP-T approach offers a number of advantages to developing-world producers, including a simple, rapid and efficient process, the absence of patent restrictions (at least for its original form designed by NICHD/NIH) and accessibility of the carrier protein [48]. The conjugation and purification process of PRP-T type conjugates is illustrated in Figure 1.

Because of the relatively complex manufacturing process, sustainable production and manufacturing of conjugate vaccines requires a cadre of well-trained individuals. As conjugate vaccines are manufactured from purified components by a defined chemical process, there is a need for standard operating procedures, meticulous attention to detail, and testing at many stages of the process. Modern physicochemical methods allow biological pharmaceuticals to be characterized with a degree of precision not previously possible. These techniques are based on the structure of the active ingredient and are thus complementary to traditional bioassays used in the industry. The techniques for analysis of conjugate vaccines include optical spectroscopy (circular dichroism and fluorescence spectroscopy), size exclusion chromatography (especially when combined with light-scattering detection techniques) and mass spectrometry; whereas the saccharide component of glycoconjugates (comprised of protein-saccharide) can be characterized by NMR spectroscopy and composition analysis performed using the sensitive technique of high-performance anion-exchange chromatography. Furthermore, it is necessary to ensure that manufacturing consistency for vaccine lots used in the clinical trials is documented, that these lots adequately represent the formulation intended for marketing and that all assay procedures used for quality control of the conjugate vaccines and vaccine intermediates are validated [49].

The additional skills acquired by local manufacturers who develop conjugate technology are likely to result in improvements in the quality of other vaccines produced in their facility.

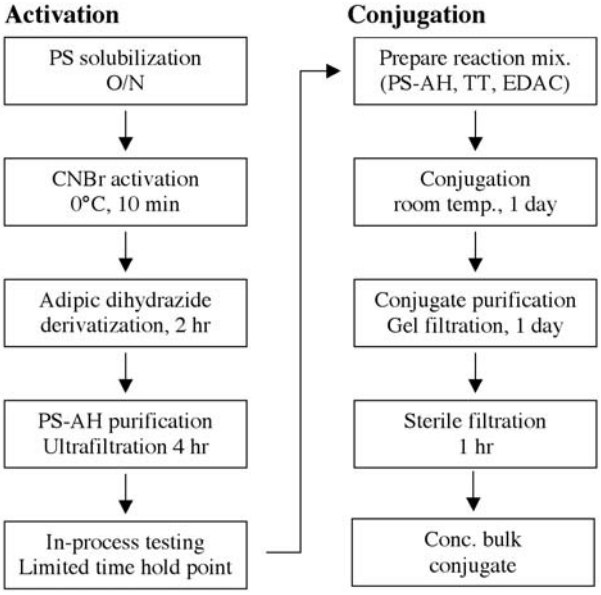

Figure 1. Conjugation and purification (PRP-T-like process)

Conjugate vaccines can be developed at reasonable cost for developing countries

The price of the vaccine is an important factor affecting its widespread use into EPI. A formal market study to assess the relationship between price and use has not been conducted, so the sensitivity of price to use cannot be formally assessed. Evidence from both WHO research and uptake of other new vaccines has repeatedly suggested that even with donor support, most developing countries struggle to finance immunization programs and have been reluctant to introduce the more expensive vaccines [4]. However, an early policymaker survey done by the International Vaccine Institute (unpublished document) revealed an unexpectedly high willingness to pay for typhoid and *Shigella* conjugate vaccines, both among the middle classes and the poor in developing countries of Asia, indicating that introduction of these vaccines through the private sector is viable, and could provide a platform for tiered pricing and provision of these vaccines to the poor at affordable prices.

A cost-of-goods analysis was undertaken by WHO in the year 2000 for the development of a group A meningococcal conjugate vaccine. In this analysis, the cost of goods (based on 2.5 million 10-dose vials) for a group A meningococcal conjugate vaccine was determined to be $0.15/dose. These

numbers are based on theoretical yields since the final yields will be determined only through the experience of producing actual lots of vaccine in the manufacturing facility. What is important is that the cost of the antigen itself accounts for only $0.04 of the final costs. Thus even if the yields were only 50% of what is expected, the cost per dose would not rise appreciably. This is a fairly simplistic analysis of costs of manufacture because some of the costs, e.g. quality control (QC) release, are related to the number of lot failures. Using the analysis of manufacturing costs of group A meningococcal conjugate, a ceiling price of $US 0.40 per dose in lyophilized ten-dose vials was set for discussions with potential partners [17].

Models for transfer of technology to local producers

The availability of affordable conjugate vaccines for developing-market diseases is more likely if more than one qualified local producer enters the market. For this to occur, selected local producers must acquire the technology and the expertise to produce conjugate vaccines, be prepared to invest in facilities to allow large-scale production and be capable of sustaining high-quality production over time.

There are several models that could potentially be used by local producers to acquire the needed technology. This section describes these models, their advantages and disadvantages.

The private sector (Big Pharma, Small Pharma and Biotech companies)

The most efficient method for local producers to acquire technology and know-how for production of conjugate vaccines would be to form an alliance with an established vaccine manufacturer with a proven track record in developing and licensing these products and a strong IP position. Big Pharma has rarely exercised this option partly because of the difficulties in obtaining a return on investment, partly because of the difficulties of working in developing countries and partly because of their insistence that the resulting products meet current (or future) FDA or EMEA standards. Where relationships have developed they largely relate to filling and finishing product/s produced in Europe and the USA.

It is most unlikely that Big Pharma would either provide a license of its technology to developing-country manufacturers for production of conjugate-based developing-market vaccines intended for local use, or, given the increasing influence of the World Trade Organization (WTO), that local producers would be prepared to use “pirated” technology.

Even when transfer of technology is contemplated, such as GlaxoSmithKline’s (GSK) agreement with Biomanguinhos for production

of DTP-HepB-Hib combinations for Latin America, the local producer is constrained from producing developing-world products based on the parent companies' IP-protected technology.

Although useful, especially for conjugate vaccines targeting global diseases, this model is not likely to result in widespread diffusion of conjugate vaccines for the poor. At best, this pattern of collaboration will be *ad hoc* and poorly suited for ensuring a global supply of needed products. Moreover, this strategy will be employed primarily for products targeted at countries in which there is a middle-class market for profitable sales, a situation that clearly excludes most of the GAVI countries. Small Pharma or Biotech companies with a proven track record for conjugate technology, but with limited capacity for production, may also consider transferring the technology to local producers to enlarge their production capacity, allowing them to become global players. But again, the same limitations cited for alliances with Big Pharma will also be applicable to these partnerships.

Public-sector institutions

In the past, government-funded in-country producers, such as the Nordic Public Health Laboratories, have attempted to produce vaccines targeting developing country populations and to transfer the technology to local producers. Because these institutions were under-resourced and lacked the capacity to scale up operations, the results have been disappointing.

Some years ago, The National Institute for Public Health and the Environment (RIVM) in the Netherlands started a Hib conjugate project that is now idle as the organization has been privatized. RIVM's technical package has been made available to several institutes which are attempting to develop the product. These institutions include BioFarma in Indonesia, Birmex in Mexico and Biological-e in India. Although work has been in progress for several years, in the absence of dedicated experienced support, none of these organizations has yet been able to develop and license a product.

It is important to analyze these efforts so that critical success factors can be identified. There are several reasons to explain why these attempts have, for the most part, failed to materialize: firstly, their priorities were almost entirely dictated by in-country needs; secondly, their small additional capacity and resources limited their flexibility to adapt their development portfolio to products targeting the developing world; thirdly, there was a lack of sustained resources for long-term technical assistance and training; and fourthly, major investments were needed at the local producer level in the developing world to make this technology transfer a success. Furthermore, increased production costs have made small-scale production unprofitable and governments have started to question the viability of such institutions. Given the current trend to privatize or close down these institutions, it is

improbable that they could fulfill this function, even if dedicated resources were provided.

Academic laboratories have also attempted to transfer the technology produced at laboratory level to producers in the developing world. The University of Gothenborg, with help from NIH and WHO, transferred the technology to produce a killed O1 oral cholera vaccine to the National Institute of Hygiene and Epidemiology (NIHE) in Vietnam [50]. The Laboratory of Developmental and Molecular Immunity at NICHD/NIH has transferred the technology for Vi conjugate and shigella conjugate vaccines to the Lanzhou and Chengdu Institutes in China, and has transferred Vi technology to Biofarma in Indonesia and the National Institute of Vaccine and Biological Substances (IVAC) in Vietnam. Unfortunately, academic institutions have limited capacity to conduct or to transfer the critical skills in in-process research and production necessary to assure successful transfer of technology for production of conjugate products for public health programs.

The dedicated not-for-profit and virtual company approaches

One way of developing vaccines against diseases that primarily occur in poor countries (where traditional market forces are insufficient to stimulate private investment) is establishing a not-for-profit (NFP) company, funded by the public sector [13]. The NFP company would be dedicated to production and licensure of one or a small number of products and not be distracted by competing projects of higher priority. A second advantage is that the NFP company would be free to design the product specifically for use in a developing-country setting, rather than adapt a product originally designed for industrialized countries. The disadvantages of this model are the need to construct and equip a dedicated facility and to recruit and retain staff with skills in all aspects of the development and production process and to establish a product pipeline which justifies the investment. As facilities are expensive and take a long time to construct and validate, and skilled staff are in scarce supply, it seems prudent to invest in manufacturers with existing facilities and staff rather than start from scratch.

Another alternative is to attempt to produce vaccines through a virtual company model as is being attempted by the International AIDS Vaccine Initiative (IAVI) [51] and the Malaria Vaccine Initiative (MVI) [15].

The MVP took a further step by attempting to develop a group A meningococcal conjugate vaccine in partnership with a developing-country vaccine manufacturer with financing, technical assistance, and coordination by the MVP. The MVP is also working with accredited National Control Laboratories (NCLs) for characterization of the vaccine and lot release, and has developed clinical and regulatory plans needed for licensure of the

vaccine in the developing country where the vaccine will be manufactured. The MVP works as both funder and project manager. It is expected that a vaccine against group A meningococcus will be available by the epidemic season of 2007–2008 [17].

It is important to note that the management of virtual organizations is very complex and that contracting out all aspects of the development process increases the risk that something will go wrong. An added risk exists when some of the partners are governmental organizations, whose priorities may be affected by external factors, such as the recent focus on bioterrorism, leading to unexpected delays. Most partner institutions with skills in serology, physicochemical characterization or lot-to-lot release are governmental institutions that prioritize national vaccine programs over international work. After September 11th, national security interests have become an urgent priority and research on conventional vaccines has shifted dramatically to research on bio-terrorism [52].

It is not surprising that the most successful models for vaccine production (e.g., Merck, Sharpe, Dohme (MSD) in the 1960–80s, GSK since the late 1980s) have brought as many of the elements of vaccine production as possible (research, development, manufacturing, clinical and regulatory affairs) under central control.

A potential solution: The IVI Center for Developing-Market Conjugate Vaccines

This paper has argued that the development of conjugate vaccines will be essential to effectively combat a wide range of bacterial diseases that account for more than three million deaths a year. It is becoming increasingly evident that additional development, production and sustainable cost-competitive supply of conjugate vaccines will only be achieved if a number of highly qualified producers in Asia and Latin America can: 1) acquire this technology; 2) produce enough numbers of doses; and 3) be appropriately trained in production, quality control/quality assurance (QC/QA) and regulatory processes. For this to happen, there are different models that can potentially be followed, ranging from an alliance with established vaccine manufactures to a complex series of partnerships with developers and subcontractors. In the long run, none of these models will achieve sustainability of affordable production of conjugates against a broad spectrum of diseases. There is a dearth of institutions with the capacity to provide transfer of conjugate technology and/or PS fermentation/purification and continuous assistance in process research, physicochemical characterization, QC/QA and lot-to-lot release to local producers. The International Vaccine Institute (IVI), an international research organization located in the Republic of Korea, with its new research laboratories occupying 211,713 ft^2 of floor space, can become such an institution. The IVI Center for

Developing-Market Conjugate Vaccines aims to combine the best practices of successful manufacturers for the production of lots of a novel vaccine and to work closely with a small number of local manufacturers to successfully transfer the technology to mass production.

The critical elements of the program are (i) a laboratory-based program of process development, where IVI scientists, overseas experts and staff from developing-country manufacturers can work together to establish production technology and transfer it to the eventual production facility (or facilities) (ii) a complementary clinical trials program to assess the safety, efficacy and effectiveness of these vaccines and (iii) support for training programs to upgrade the skills from National Regulatory Authorities and NCLs from developing countries.

To achieve these goals, long-term financial and political commitment is needed. The GAVI and the public sector at large have an extraordinary opportunity to leverage the considerable resources and personnel already invested in the IVI to establish an operational arm at the IVI to accelerate the development and introduction of new-generation conjugate vaccines for developing-country populations.

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Novel vaccination strategies

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Introduction

Vaccination is one of the most significant public health interventions ever made, sparing millions of people from infectious diseases. It has been calculated that use of currently available vaccines saves more than eight million lives annually, which corresponds to one person saved every five seconds. These remarkable results have been achieved in 200 years of vaccination history, which are largely based on two basic, simple technologies: the inactivation of toxins and pathogens with formaldehyde treatment (first performed by Glenny and Hopkins in 1923 and Ramon in 1924) [1–3], and the stable attenuation of pathogens by serial passage *in vitro*.

From 1920 to 1980, these now "easy technologies" were applied to develop vaccines that have been used worldwide to control or even eradicate many infectious diseases. One of the most brilliant successes of vaccination is the eradication of smallpox virus in 1977. It has been estimated that to date, smallpox eradication has spared the global community of some 350 million new smallpox victims and about 40 million deaths from the disease. The annual savings as a result of vaccination being stopped and hospitals being able to be converted to other uses is estimated to be in excess of US$ 2000 million each year [4].

Vaccination has also resulted in the elimination of poliovirus from the Americas in 1994, Western Pacific in 2000 and Europe in June 2002. The goal of the WHO Global Polio Eradication Initiative is to interrupt transmission of wild poliovirus by end-2004 leading to global certification by 2008. Overall, since the Initiative was launched in 1988, the number of cases has fallen by over 99%, from an estimated 350,000 cases to the 89 cases reported in the first three months of 2004. In the same period, the number of polio-infected countries was reduced from 125 to 6 [5].

Moreover, the incidence of seven other frightening diseases (diphtheria, measles, rubella, mumps, pertussis, *Haemophilus influenzae* type b – Hib –, and tetanus) has fallen by more than 98% in those states where the related vaccines have been introduced (see Tab. 1).

Table 1. Universal routine vaccination reduced by more than 98% the incidence of nine harmful diseases from the US and eradicated two of them (smallpox and polio).

Disease	Max number of cases (year)	Number of cases in 2002	Reduction (%)
Smallpox	48,164 (1901–1904)	0	100
Poliomyelitis	21,269 (1952)	0	100
Diphtheria	206,939 (1921)	1	99.99
Measles	894,134 (1941)	44	99.9
Rubella	57,686 (1969)	18	99.78
Mumps	152,209 (1968)	270	99.86
Pertussis	265,269 (1934)	9771	98.20
H. influenzae type b	20,000 (1992)	<100	98.79
Tetanus	1,560 (1923)	25	98.44

Source: CDC (1998) Impact of vaccines universally recommended for children – US, 1990–98. *MMWR* 48: 577-581; modified) [94].

Novel strategies for novel vaccines

The historical technologies for vaccine production have achieved remarkable successes, but the battle against infectious diseases has not been won. During the last several million years of existence on earth, microorganisms have learnt how to escape the surveillance of our immune system, either hiding themselves from host responses, or developing the capacity of infecting different hosts. These two main mechanisms of pathogen defense explain why vaccines against several pathogens such as HIV, HCV, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Plasmodium falciparum*, *Schistosoma* spp. and others are difficult to develop and are not yet available.

Moreover, during the last 25 years, there has been a big revolution in microbial ecology: pathogens that have circulated since many years, but whose existence was ignored were discovered (HCV, MetaPneumoVirus, etc.); old pathogens have changed their geographical habitat and have been introduced into new areas where they have become endemic (e.g. West Nile Virus in North America); completely new pathogens have emerged and pose a serious threat to human health (HIV, SARS Coronavirus, etc); pathogens thought to be controlled have re-emerged (*Mycobacterium tuberculosis*, etc.) (Tab. 2).

It was soon clear that the old vaccine strategies were unable to face with the new challenges posed by infectious diseases, and modern approaches were needed. Therefore, from the beginning of the 1980s, taking advantage of the better understanding of the immune system and the emergence of the recombinant DNA technologies, new vaccines were developed (Tab. 3). Moreover, nowadays the range of technologies and strategies available for vaccine development is extremely broad. The availability of the whole genomic sequences of many pathogens is considered a real revolution and

Table 2. New and emerging pathogens identified in the last 25 years

Period	Pathogen
1980–1990	HTLV HIV *Escherichia coli* O157H7 *Borrelia burgdorferi* *Helicobacter pylori* Human herpesvirus type 6 *Ehrlichia chalffeensis*
1990–2004	HCV *Vibrio cholerae* O139 *Bartonella* spp. Hantavirus Nipah virus West Nile virus TT virus Meta Pneumo virus SARS Coronavirus

a start of a new era in the vaccine field. Genomics, proteomics, molecular biology, recombinant DNA, and advanced immunology have removed most of the technical barriers that formerly limited vaccine development approaches to prevent and not only treat infectious diseases, but eventually also cancer, allergies, and other chronic conditions.

Conjugate vaccines

It is now common knowledge that the capsular polysaccharide of many important bacterial pathogens is their major virulence factor and that vaccination using purified polysaccharides could protect people from those diseases. Polysaccharide vaccines have been developed against *Neisseria meningitidis* serogroup A (MenA), group C (Men C), group Y (MenY), and group W135 (Men W135) in the late 1960s [6, 7], against *H. influenzae* type b in the 1980s [8], against 23 types of *Streptococcus pneumoniae* in the early 1990s [9], and against *Salmonella typhi* in the mid 1990s [10]. However, the use of all these vaccines is inadequate. Polysaccharides are T-independent antigens: their immunogenicity and efficacy is very poor or absent in infants, so that they are unsuitable to be included in the infant routine immunization schedule.

The solution was found by covalently linking the sugar to a carrier protein (conjugation). This procedure converts T-independent antigens into T-dependent ones by providing a source of appropriate T-cell epitopes (present in the carrier protein). Conjugation technology is one of the biggest achievements of the novel vaccination strategies. The capacity of transforming poor immunogens such as polysaccharides into excellent immuno-

Table 3. Vaccines licensed after 1980

Disease	Year
Hepatitis B (HBV)	1986
Haemophilus influenzae type b, conjugate	1990
Salmonella typhi (live attenuated, capsular polysaccharide)	1991
Hepatitis A (HAV)	1994
Varicella	1995
Acellular pertussis (aP)	1996
Influenza (adjuvanted)	1997
Lyme disease	1998
Neisseria meningitidis group C conjugated	2000
Streptococcus pneumoniae 7 valent conjugated	2000

gens such as the protein-sugar complex has provided outstanding tools for the fight against infectious diseases.

Haemophilus influenzae type b (Hib)

The first conjugate vaccine developed was the one against *Haemophilus influenzae* type b (Hib) [11]. Hib is a respiratory pathogen that causes a wide spectrum of human infections. Before the availability of the Hib conjugate vaccine, Hib was the leading cause of bacterial meningitis, especially in infants, in most developed and developing countries worldwide, resulting in substantial morbidity and mortality. The first Hib conjugated vaccine was introduced in the US in December 1987 for children 18 months of age and older; subsequently, more immunogenic vaccines were introduced, in US as well as in many other countries, in the early 1990s, for the use in infants from the age of two months.

Within a few years, the effectiveness and public impact of Hib conjugate vaccines on Hib disease has been demonstrated in all those countries that have introduced the vaccination [12]. In the US, the annual cases of Hib invasive disease among children under five years of age decreased from more than 20,000 of the pre-vaccination era [13], to less than 50 in 2002 [14].

In general, the decrease in incidence of disease has exceeded that predicted on the basis of estimated proportion of the population that is completely immunized, suggesting that the vaccine use is able to generate herd immunity. On this topic, many studies have shown that Hib conjugated vaccines are able to reduce nasopharyngeal carriage [15–18], which gives a major contribution to herd immunity. Precisely how Hib conjugate immunization reduces nasopharyngeal colonization, whether this impact is due to decreased acquisition or decreased duration of carriage is not clear. Additional work on the immunology and microbiology of transmission and carriage is needed to clarify this issue. On the other hand, reduction of Hib carriage may open ecological niches for *H. influenzae* non-type b strains

and therefore potentially increase the risk of colonization and invasive disease by other dangerous strains, for which a vaccine is not available [19–21]. However, so far, widespread vaccination against Hib has not caused this problem: strains recovered from vaccinated children are indistinguishable from those recovered from unvaccinated ones [22].

Streptococcus pneumoniae

The first conjugate vaccine licensed for human use, initially in US in February 2000, and subsequently in Europe, contains capsular polysaccharides of seven serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) conjugated to the CRM197 as carrier protein by reductive amination. Other vaccines under evaluation cover nine and 11 serotypes. However, due to practical and economic reasons, only a limited number of serotypes could be included in conjugate vaccine formulations [23]. The choice of the strains should be based to target the serotypes that most commonly cause diseases among population and the geographic region to be vaccinated [24]. The seven serotypes in the first licensed conjugate vaccine account for >80% of isolates from blood and cerebrospinal fluid of children aged <2 years in the US, compared with <60% of isolates from older children and adults in US, and with <60% of isolates from young children in Latin America and Asia [25, 26].

The seven-valent conjugate pneumococcal vaccine has been recommended in US for all infants as three primary doses (approximately at 2, 4, and 6 months of age) and one booster dose at the ages of 12 and 15 months [27]. This vaccine repeated the successes of the Hib conjugate vaccine, showing high efficacy in preventing meningitis and efficacy, although to less extent, in preventing pneumonia [28] and acute otitis media [29] due to the vaccine serotypes. Moreover, several studies have established that the conjugate vaccine is effective also in reducing nasopharyngeal carriage of the serotypes covered by the vaccine, potentially extending the benefits beyond those children who are vaccinated. Post licensure evaluation of the efficacy of the seven-valent conjugated vaccine found that the reduction in disease was greater than the percentage of children who have been vaccinated, with evidence of herd immunity [30].

Neisseria meningitidis serogroup C

Neisseria meningitis group C conjugate vaccines began clinical development at the end of the 1980s, and, after extensive clinical studies during the 1990s, were proved to be safe and capable of inducing highly bactericidal and boostable immune responses in infants and children [31–39]. These vaccines were first introduced in the United Kingdom in late 1999, to be used

for a countrywide vaccination initiative. Population-based active surveillance monitored age-specific and capsular group-specific incidence of disease and estimated vaccine coverage [40]. The results were impressive: within 18 months of vaccine introduction, the cases of MenC diseases almost disappeared in the vaccinated population, with an estimated vaccine efficacy of 90%, or above, in all targeted age groups [41]. According to a large carriage study performed in the United Kingdom, the vaccine induced also a 66% decrease in the carriage of MenC strain, with no evidence of any increase in carriage of MenB isolates or other capsular groups [42]. Moreover, the vaccine provides some evidence of herd immunity, as there is also a reduction of MenC disease in unvaccinated individuals ranging from 34 percent (9–14 years old) to 61 percent (15–17 years old) [43].

Since 1999, the MenC conjugate vaccine has been licensed in many European countries and also in Canada and Australia, either as routine infant immunization, or for catch-up programs of varying forms.

Multivalent meningococcal conjugate vaccines containing groups A, C, Y, and W135 are in clinical development. Introduction of these vaccines into routine infant and/or toddler immunization programs could have a substantial effect on the incidence of meningococcal disease [44, 45].

Recombinant DNA for subunit vaccines: HBV example

In the 1970s it was discovered that protection towards hepatitis B is correlated to the presence of antibodies against the surface antigen of the virus (HBsAg), which circulates in the bloodstream of infected subjects in large quantities. These discoveries led to the development of first generation, plasma-derived HBsAg subunit vaccines, first licensed in the US in 1982 [46]. Concerns about safety of blood products and the advances of recombinant DNA technology conducted to the development of second-generation recombinant subunit vaccines produced in yeast or in mammalian cells [47], which were first licensed in US in 1986. The application of the recombinant DNA technology has the advantage to produce safer vaccines than the plasma- derived ones and in unlimited supplies. This recombinant HBV vaccine is highly effective: a standard course of three doses induces protective levels of anti-HBs (i.e. >10 mIU/ml) in over 95% of healthy infants, children and adolescents and in more than 90% of healthy adults younger than 40 years. After the age of 40, immunogenicity drops below 90%: however, and by the age of 60 years, 65 to 75% of vaccinees develop protective anti HBs titers [48–50]. Moreover, the vaccine prevents development of chronic hepatitis in at least 75% of early-vaccinated infants born from HBeAg-positive mothers [51].

Cost-benefit analyses have strongly supported the introduction of universal immunization against HBV to newborns [52]. Since 1992, the WHO has called for all countries to add hepatitis B vaccine into their national

childhood immunization schedules, and substantial progress has been made in implementing this recommendation: by the end of 2003 more than 160 countries worldwide will have introduced the vaccine [53]. Substantial help in achieving this goal has been given by the Global Alliance for Vaccines and Immunization (GAVI) and the Vaccine Fund, which since 2000 began to provide technical and financial support to introduce routine HBV immunization into 71 of the world's poorest countries [54].

The effectiveness of routine infant HBV immunization in significantly reducing or eliminating the prevalence of chronic HBV infection has been demonstrated in a variety of countries and settings. The experience in Taiwan is particularly impressive. Universal vaccination decreased the prevalence of HBsAg carriage in children younger than 15 years of age from 9.8% in 1984 to 0.7% in 1999 [55]. Moreover, rates of hepatocellular carcinoma (HCC) among cohorts of children born after routine infant immunization have started in the mid 1980s declined by greater than 50% [56]. These results clearly show that the HBV vaccine is the first successful anti-cancer vaccine, opening the way to other anti-cancer vaccines like vaccines against human papilloma virus (HPV) that causes cervical cancer [57], or *Helicobacter pylori*, responsible for many gastric cancers [58].

Genetically detoxified vaccines

The first example of a vaccine developed with a new rational strategic design, taking advantage of the power of the molecular biology and genomic technologies is the recombinant acellular pertussis vaccine.

A vaccine composed of the whole, killed *Bordetella pertussis* cells has been available for mass vaccination since the late 1940s [59]. This vaccine is very efficacious in preventing the disease, but the presence of severe adverse reactions, although no proof exists of their having been caused by the vaccine, brought about a drop in vaccine compliance in the 1970s and stressed the need for a new safer vaccine.

A considerable amount of work was initially required to identify the bacterial antigens responsible for the induction of protection. Pertussis toxin (PT) was then identified as a major protective antigen, while other antigens, such as adenylate cyclase, filamentous haemagglutinin (FHA), pertactin and the fimbriae, were found to provide some help in protective immunity. Several researchers developed acellular pertussis vaccines containing purified PT inactivated by the old classical chemical treatment with formaldehyde and glutaraldehyde. However, it is recognized that this treatment of PT may be associated with significant reversion rates [60].

To overcome these problems, the pertussis toxin was detoxified by using the powerful tools of molecular biology to make stable changes, by site-directed mutagenesis, to the amino acids responsible for the toxicity of pertussis toxin. This molecular approach allowed the development of a natu-

rally nontoxic molecule, inactivated by a rational design that did not need a denaturing chemical treatment [61].

The genetically detoxified PT had successfully shown its safety and immunogenicity in clinical trials, both in adult volunteers and in infants and children, either as a monovalent mutant PT alone [62, 63], or in association with FHA and pertactin [64, 65], and also with FHA and pertactin in association with diphtheria and tetanus toxoids (DTaP) [66, 67]. These trials showed that the different acellular vaccine formulations containing the non-toxic PT mutant were extremely safe, and much safer than the whole-cell pertussis vaccines. Furthermore, all formulations induced high titers of anti-PT neutralizing antibodies and very strong antigen-specific T-cell proliferative responses. Interestingly enough, five to six years after the primary immunization schedule, this vaccine still exhibited an efficacy of about 80%, and both antigen-specific antibody and $CD4^+$ T-cell responses were still detectable at significant levels [68, 69].

The new strategy used to develop this genetically detoxified acellular pertussis vaccine shows that this approach can provide tremendous advantages, eliciting very high, protective immune responses with very low amounts of antigen.

The genomic approach: reverse vaccinology

Despite the introduction of these new strategies (conjugation, recombinant DNA etc.), one of the major issues for the development of new vaccines is the identification of protective antigens from a microorganism. Biochemical, immunological and microbiological methods have been successful in many cases, but they require the pathogen to be grown in laboratory conditions, are time-consuming and allow for the identification only of the most abundant antigens, which can be purified in quantities suitable for vaccine testing. Unfortunately, in many instances the most abundant proteins could not be good vaccine candidates. Moreover, the antigens expressed *in vivo* during the infection could not be expressed *in vitro* to the same extent. Furthermore, when dealing with non-cultivable pathogens, there is no approach to vaccine development.

The availability of the complete genome sequence of a free-living organism (*H. influenzae*) [70] marked the beginning of the "genomic era" that opened novel strategies in vaccine design. The possibility of determining the whole sequence of a genome (bacterial, viral or parasitic) led to the idea of using the genomic information to discover novel antigens that have been missed by conventional vaccinology. This approach, called "reverse vaccinology", involves the computer (*in silico*) analysis of the microbial genome sequence and predicts those antigens that are most likely to be vaccine candidates [71, 72]. The success of genomic-based strategies for vaccine development is highly dependent on the criteria used for the *in silico* selection of

the potential antigens, which are usually secreted or extracellular proteins, more easily accessible to antibodies than intracellular ones. Several approaches can be used to mine genomic sequences, and the appropriate combination of various algorithms and the critical evaluation of the information generated are essential for the proper selection of the antigens.

The *in silico* approach results in the selection of a large number of genes. Therefore, it is necessary to use simple procedures that allow large numbers of genes to be cloned and expressed, like robotics and PCR. The product of each PCR reaction is cloned and screened for expression in a heterologous system. Successful expression depends on the predicted localization of the protein. Integral transmembrane proteins have proven to be particularly difficult to produce by recombinant techniques in *E. coli*.

Once purified, the recombinant proteins are used to immunize mice and the post-immunization sera are analyzed to verify the computer-predicted surface localization of each polypeptide and their ability to elicit a quantitative and qualitative immune, protective response.

The genomic approach has many novel features and advantages. There is no need to cultivate the pathogen. As the whole process starts *in silico*, it could be equally applied to cultivable and non-cultivable microorganisms. Pathogens dangerous to handle can be studied as easily as commensals. Virtually all protein antigens encoded by the pathogen's genome could become vaccine candidates, regardless whether they are expressed *in vitro* or *in vivo*, abundant or scarce. This process allows the identification of all the antigens seen by the conventional methods and the discovery of novel antigens that work on a totally different paradigm.

Unfortunately, reverse vaccinology suffers from limitations related to our incomplete knowledge of vaccine immunology: a rate-limiting step of the process is linked to the rarity (or, more often, absence) of widely accepted correlates of protection and reliable animal models of infection. The other constraint of this approach is the inability to identify non-protein antigens such as polysaccharides, and CD-1 restricted antigens such as glycolipids, which represent new promising vaccine candidates.

Meningococcus B

Meningococcus B represents the first example of the application of the genomic approach and the demonstration of the power of this novel strategy for target antigen identification [73]. Conventional approaches to MenB vaccine development have been ineffective for more than 40 years. A capsular polysaccharide-based approach, which has been extremely successful for other major meningococcal serogroups, cannot be used for this strain, because the MenB capsular polysaccharide is identical to a widely distributed human carbohydrate (α(2–8)N-acetyl neuraminic acid, or polysialic acid). This condition not only makes the polysaccharide a poor

immunogen for humans, but also a potential generator of autoantibodies. On the other hand, a protein-based approach, developed using surface-exposed proteins contained in outer membrane vesicles (OMVs), has shown to have efficacy in clinical trials only against homologous strains, especially in children below five years of age, because of the high sequence variability of the major protein antigens [74].

Using the reverse vaccinology, DNA fragments were screened by computer analysis while the *N. meningitidis* sequencing project was in progress. The screening was aimed to select proteins predicted to be on the surface of the bacterium or to have homologies to known bacterial factors involved in pathogenesis and virulence. After clearance of cytoplasmic proteins and known *Neisseria* antigens, 570 genes predicted to code for surface-exposed or membrane-associated proteins were recognized. Successful cloning in *E. coli* and expression was achieved for 350 proteins, which were then purified and tested for localization, immunogenicity and protective efficacy in animal models. Of the 85 proteins found to be surface exposed, 22 were able to induce complement-mediated bactericidal antibody response, which is the most convincing indication of the capability of inducing protective immunity. In addition, to test the appropriateness of these proteins as candidate antigens for conferring protection against heterologous strains, the proteins were evaluated for gene presence, phase variation and sequence conservation in a panel of genetically diverse MenB strains representative of the global diversity of the natural *N. meningitidis* population. Most of the selected antigens were able to induce cross-protection against heterologous strains, demonstrating that these new antigens are good candidates for the clinical development of a vaccine against MenB [73, 75].

It is impressive to notice that within only 18 months the genomic approach allowed the discovery of more potential vaccine candidates than the previous 40 years of conventional research.

The success of the MenB project, together with the availability of an increasing number of genomic sequences and the faster and faster development of bioinformatics tools, has encouraged the application of the reverse vaccinology approach to many other pathogens. Nowadays, this approach is widely used to develop vaccine candidates against those pathogens for which conventional approaches have failed so far. The potential of the genomic approach is so extensive that it could be used not only for bacteria, but also for viruses and parasites.

Streptococcus pneumoniae

Despite the successes of the conjugate vaccine against *Streptococcus pneumoniae* (see above), the absence of cross protection between the different serotypes, with the threat of serotype replacement by strains that are not represented in the vaccine composition, makes it unsuitable for universal

use [20]. To identify more appropriate vaccine candidates, the whole genome sequence of *S. pneumoniae* was scanned and 130 potential ORFs with significant homology to surface proteins and virulence factors of other bacteria were identified [76]. 108 of these proteins were effectively expressed in *E. coli* and used to immunize mice; six of them were able to confer protection against pneumococcal challenge in a mouse sepsis model. Flow cytometry confirmed the surface localization of these new proteins. Furthermore, each of the six protective antigens showed a high degree of cross reactivity against the majority of capsular antigens that are expressed *in vivo* and are immunogenic during human infection, providing a good base for the development of improved vaccines against *S. pneumoniae*.

Streptococcus agalactiae

Streptococcus agalactiae, or group B streptococcus, is the leading cause of bacterial sepsis, pneumonia, and meningitis in neonates in US and Europe [77]. As for *S. pneumoniae*, the capsular polysaccharide is the major protective antigen, but at least nine different seroptypes exist with little or no cross protection [78]. Therefore, a protein-based vaccine is required to overcome serotype differences.

The complete genome of a serotype V strain of *S. agalactiae* was determined and analyzed [79]. The genome is predicted to code for 2175 ORFs of which 650 were predicted to be exposed on the surface of the bacteria. Approximately 350 of these ORFs were successfully expressed in *E. coli* and used to immunize mice. Using the sera in ELISA and flow cytometric analysis against intact bacteria, it has been demonstrated that 55 of these proteins are in fact measurably expressed on the surface of the bacterium. These new antigens are now being evaluated in *in vitro* and *in vivo* models for their capacity to protect against invasive infection by group B streptococcus.

Staphylococcus aureus

To identify vaccine candidates for *Staphylococcus aureus*, an approach based on genomic peptide libraries and well-characterized human sera was developed [80]. *S. aureus* peptides were displayed on the surface of *E. coli via* fusion to one or two outer membrane proteins (LamB and FhuA) and probed with sera selected for high antibody titers and opsonic activity. The exhaustive screening of the two different peptide expression libraries by the application of MACS technology (magnetic cell sorting) enabled the profile of antigens that are expressed *in vivo* and that are able to elicit an immune response in humans to be identified. A total of 60 antigenic proteins were identified, most of which were predicted to be secreted, or located on the surface of the bacterium, and their antibody-binding

sites were mapped. These antigens represent promising vaccine candidates for further evaluation.

Porphyromonas gingivalis

A process very similar to the one used to develop a vaccine against MenB was used also for *Porphyromonas gingivalis*, a pathogen implicated in the etiology of chronic adult periodontitis [81]. From a genomic sequence of 2000 genes, 120 were selected using a series of bioinformatics tools. The selected genes were cloned for expression in *E. coli* and screened by Western blotting using sera from human periodontitis patients and animal antisera. This subset of 40 recombinant proteins were then purified and used to immunize mice, which were subsequently challenged with live bacteria in a subcutaneous abscess model. Two of these recombinant proteins (showing homology to *Pseudomonas* sp. OprF protein) demonstrated significant protection in the animal model and therefore could represent potential vaccine candidates [82].

Chlamydia pneumoniae

Chlamydia pneumoniae is an obligate intracellular parasite with a complex biphasic lifecycle: an extracellular infectious phase characterized by a spore-like form, the elementary bodies (EB), and an intracellular replicating stage characterized by the reticular bodies (RB). The pathogen is a common cause of community-acquired acute respiratory infections and more recently has also been associated with atherosclerotic cardiovascular disease [83].

Because of the technical difficulties in working with *C. pneumoniae* and the absence of reliable tools for genetic manipulation, not much is known about the cell surface composition of the EB. To define the surface protein organization of *C. pneumoniae*, a systematic genomic and proteomic approach was used [84]. The approach is based on six main experimental steps: (i) *in silico* analysis of the *C. pneumoniae* genome sequence to identify genes potentially encoding surface proteins (including outer and inner membrane and periplasmic proteins); (ii) cloning, expression, and purification of selected candidates; (iii) use of purified antigens to generate mouse immune sera; (iv) analysis of sera specificity by Western blotting of total EB extracts; (v) assessment of antigen localization by FACS analysis on whole EBs; and (vi) identification of FACS-positive antigens on bidimensional electrophoresis (2DE) maps of *C. pneumoniae* EB proteins. The results of this systematic genome-proteome approach represent the first successful attempt to define surface protein organization of *C. pneumoniae* and raise the possibility to find suitable candidates for a purified vaccine.

Reverse vaccinology for bioterrorism-associated agents: Bacillus anthracis and Yersinia pestis

Currently licensed anthrax vaccines have been developed during the 1960s and are based on cell-free filtrates containing mainly the Protective Antigen (PA). PA is the common cell-binding domain capable of interacting with two different domains, the lethal and the edema factors, which elicit cell damage. These vaccines, originally licensed for selected veterinary personnel, textile workers and abattoir workers, have been widely used for the first time for the US military during the 1990–91 Desert Storm campaign. However, this extended use has raised issues and controversies regarding safety, efficacy, and an unsatisfactory dosing regimen, as the vaccine requires 6-dose series of injections and annual boosters [85, 86].

Therefore, identification of novel antigens is essential for the development of second-generation *B. anthracis* vaccines. ORF products similar to proteins involved in bacterial pathogenesis, and secreted and surface exposed proteins are the focus of the research. Using functional genomic analysis, 11 candidates were selected. In this case, a simple method that relied on the *in vitro* translation of the linear full-length DNA of the selected ORFs was used. Polypeptides obtained *in vitro* were then evaluated for immunogenicity by analysis of their reactivity with hyperimmune anti-*B. anthracis* antisera. The combination of bioinformatic genomic analysis and an efficient and fast screening facilitated the identification of unknown antigenic proteins, three of which appear to be similar to immunogenic PA. These new proteins could represent parts of a second-generation anthrax vaccine [87].

Old plague vaccines are based on killed whole cell preparations [86, 88]. Although they appeared to be effective against endemic plague, they were not considered to be protective against respiratory plague, which is the form of most concern in case of biological attack. Moreover, these vaccines have high rates of reactions, both systemic and local, especially with successive doses and suffer from short duration of protection, requiring biannual boosters. A live-attenuated vaccine (EV76 strain) also existed. While this vaccine seemed to be effective, it retained some virulence and in most countries it was considered not suitable for human use.

In a search for novel attenuated vaccine candidates for use against *Y. pestis*, a signature-tagged mutagenesis (STM) strategy was used [89]. A library of tagged mutants of the virulent *Y. pestis* Kimberley53 strain was generated. Screening of 300 mutants through two consecutive cycles resulted in selection of 16 mutant strains. Characterization of the phenotypes and genotypes of the selected mutants led to identification of virulence-associated genes coding for factors involved in global bacterial physiology (e.g., *purH*, *purK*, *dnaE*, and *greA*) or for hypothetical polypeptides, as well as for the virulence regulator gene *lcrF*. One of the avirulent mutant strains was found to be disrupted in the *pcm* locus, which is presumably involved in the

bacterial response to environmental stress. This Kimberley*53pcm* mutant was superior to the EV76 live vaccine strain in an animal model in which mice were infected subcutaneously with the virulent *Y. pestis* Kimberley53 strain, either because the mutant induced 10- to 100-fold-higher antibody titers to the protective V and F1 antigens and because it conferred efficacious protective immunity. These results indicate that mutants with mutations in the *pcm* locus can serve as a platform for generation of a novel live vaccine with more promise for inducing efficacious protective immunity to virulent *Y. pestis* strains than previously suggested mutants.

New strategies for new diseases: SARS example

The SARS epidemic has been the first infectious disease outbreak to fully benefit from the revolutionary technologies of the post-genomic era. Thanks to an unprecedented collaboration effort between scientists from many countries, coordinated by WHO and including clinical, epidemiological and laboratory investigations, within 15 days from the issue of the global alert, the causative agent of the new disease was discovered [90]. In less than a month after the initial identification of a new coronavirus (called SARS-CoV) as the infectious agent of SARS, two independent genome sequences of the virus had been obtained [91, 92]. Within three months, the genome sequences of 20 independent clinical isolates were made available in the GenBank database. The rapid availability of the genomic sequence of the new virus has been very important from the public health perspective, giving prompt answers to a number of critical questions. It was clear that the agent was a natural (and not a laboratory-fabricated) coronavirus, diagnostic kits were set up, and possible drug and vaccine targets identified.

Today, some of these vaccines, based on a killed virus, are already being tested in pre-clinical and even in clinical trials, but their efficacy needs to be shown. Moreover, in addition to the traditional approach, a number of newer strategies are being used. These include subunit vaccines containing recombinant spike protein expressed in mammalian cells or yeast, either alone or in combination with other SARS-CoV antigens. Alternatively, these antigens could be delivered by DNA immunization by non-replicating viruses, or viral vectors that are based on adenovirus, canarypox, modified vaccinia virus Ankara (MVA) or alphavirus. In particular, the development of non-replicating coronavirus-like particles that mimic the structure of native virions could prove promising in the search for a successful vaccine as they display a large repertoire of antigenic sites and discontinuous epitopes [93].

It is crucial to remember that none of these approaches would have been possible in such a way and with such a speed without the knowledge of the complete genomic sequence of the virus. To make a comparison, the

last time mankind faced the appearance of a new pandemic was in the early 1980s, with the appearance of HIV: at that time, only 20 years ago, it took two years to identify the etiologic agent of the infection, and a safe and effective vaccine is still lacking.

Conclusion

When Edward Jenner gave birth to the concept of vaccination little could he know that after more than 200 years mankind would still be battling against emerging and re-emerging infectious diseases. However, contemporary society is lucky in that science is constantly offering more effective tools to combat and control infectious diseases. The question is whether the many social, political, cultural, and economic barriers that still exist *vis-à-vis* vaccines will be eventually overcome, thus recognizing the real value of vaccination.

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Design and selection of vaccine adjuvants: principles and practice

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The requirement for vaccine adjuvants

Experience has shown that the initiation, quality, and magnitude of the immune response to a vaccine antigen can be influenced by numerous factors. These include the type and dose of the antigen, the timing of immunization and boosting, the route of immunization, and even the age and general health of the vaccinee.

Addition of an adjuvant to the vaccine formulation to enhance the immune response in a safe and nonspecific manner has also been a strategy that has been successfully employed for many vaccines. In past years, and even to the present, this has been accomplished mainly through adsorption of the antigen to an aluminum salt [1–3]. However, it should be noted that aluminum salts generally perform only weakly as adjuvants, particularly for booster immunizations, even though they do have a relatively good record of safety.

Modern research has demonstrated that different adjuvants can have profound effects not only on the magnitude of the immune response, but also on the qualitative type of immune response. Two illustrative examples of this are given below. In the first example, among many that could be cited, differences in protection against *Mycobacterium tuberculosis* challenge in mice were induced by different adjuvant formulations [4]. In this study it was found that injection of recombinant mycobacterial 65 kDa heat shock protein (hsp65) as an antigen by itself was unable to protect mice, even with Freund's incomplete adjuvant. However, when the antigen was administered as a DNA vaccine or as a recombinant protein entrapped in cationic liposomes, protection was observed. In contrast, when hsp65 was encapsulated in polymeric microspheres for sustained release of antigen, high levels of specific antibody were observed but the mice were not protected after challenge. As the authors stated, the vaccine adjuvant "makes

the difference." In another illustrative example of differences induced by different combinations of adjuvants, it was shown that interleukin-12 (IL-12) together with granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumor necrosis factor-alpha emulsified in Freund's adjuvant could be used to steer immune responses to induction of cytotoxic T lymphocytes [5]. GM-CSF alone gave mixed Th1 and Th2 responses, while GM-CSF plus IL-7 gave predominantly a Th2 response in BALB/c mice.

Inclusion of an adjuvant is not always necessary in order to achieve a successful vaccine effect. Sometimes a highly purified vaccine antigen by itself has inherent adjuvant activity. Cholera toxin (CT) from *Vibrio cholerae* or heat-labile enterotoxin (LT) from *Escherichia coli* are examples of purified antigens that can exhibit both antigenic and adjuvant activity [6–9], and CT directly activates dendritic cells [10]. More commonly, however, particularly in the past, a partly purified vaccine antigen has been used that also contains a contaminating adjuvant compound or that has adjuvant activity that has not been removed by further purification. The original smallpox vaccine, containing pus from cow pox sores, represents a quintessential example of utilization of a crude semi-purified viral antigen surrounded by its own contaminating adjuvant materials. Among other examples of crude antigen preparations are included parenterally-injected killed inactivated whole cell bacterial vaccines, such as inactivated whole cell *Bordetella pertussis*, *Salmonella typhi*, or *Vibrio cholerae* vaccines, which may be contaminated with bacterial endotoxin [11]. Endotoxin is a compound that can have strong natural adjuvant activity, and all of the adjuvant activity of endotoxin is due to the lipid A moiety of the bacterial surface lipopolysaccharide.

New generations of highly purified or synthetic antigens rarely have any inherent adjuvant activity, and the low immunogenicity of most highly purified antigens has highlighted the need to include adjuvants in vaccine formulations. It may be important for certain vaccines, particularly those that address difficult diseases, such as malaria or HIV infection, to include modern adjuvants that are much stronger than the weak adjuvant activity obtained from simple adsorption of antigen to aluminum salts.

Historical adjuvants

Freund's adjuvant

Perhaps the most famous adjuvant formulations are the water-in-oil emulsions devised by Jules Freund which are commonly designated as either complete or incomplete Freund's adjuvant [12, 13]. Although complete Freund's, containing mycobacteria, is universally regarded as too toxic for human use, incomplete Freund's (lacking mycobacteria) has been used as a potent adjuvant formulation for a diversity of vaccines in more than a mil-

lion people [12, 14, 15]. It is currently being developed for certain human and veterinary vaccines [13].

Unfortunately, incomplete Freund's adjuvant has a widespread, albeit mainly undeserved, reputation for toxicity in humans [13, 14]. The reputation of incomplete Freund's adjuvant for toxicity is probably largely undeserved because it is based mainly on faulty animal models that do not reflect or translate to expression of human toxicity [15, 16]. Nonetheless, acceptance of vaccines is greatly dependent on the positive or negative perceptions that exist among potential recipients of vaccines, and because of fears of toxicity of Freund's adjuvant new generations of oil-in-water (rather than water-in-oil) vaccines have been devised that retain adjuvant activity but have essentially no toxicity in humans [17–19].

Complete Freund's adjuvant is also notable for having the dubious distinction of having created an unwarranted public fear that adjuvants might cause arthritis in humans. Adjuvant-induced arthritis, also often called adjuvant arthritis, is a condition that is uniquely associated with rodents, and is produced mainly after injection of complete Freund's adjuvant [20, 21]. The pathogenesis of the arthritogenic effects in rodents is strongly associated with a mycobacterial 65-kDa heat shock protein (Bhsp65) [22]. However, certain rodents can be so sensitive to the arthritogenic effects of Freund's adjuvant that arthritis can be induced even by painting the oil of incomplete Freund's adjuvant on the skin of genetically susceptible rats [23].

Although the rodent adjuvant arthritis model is interesting and has proven to be a boon to researchers interested in experimental rheumatology and in autoimmunity models in rodents, and is widely viewed as a model that illustrates the characteristics of rheumatoid arthritis in humans, and even for autoimmune disease in general [22], the induction of arthritis by adjuvants occurs only in rodents. Adjuvant-induced arthritis has never been recognized as an established pathological condition in humans and it therefore does not exist as a recognized human syndrome or disease entity. In a famous study in 1951–1953 Jonas Salk injected 18,000 American soldiers with an influenza vaccine that contained incomplete Freund's adjuvant, along with 26,000 control soldiers who did not receive adjuvant [24]. As reviewed earlier [15, 16], because the continuing health records of these Korean War veteran cohorts were available due to the excellent continuity of the military and veterans' medical health care system, extensive follow-up analyses after 9 years [25], 16–18 years [26], and >35 years [27] were undertaken. When records were examined for: mortality; separation or retirement from the military for disability; and for hospitalization and morbidity, no statistically significant evidence of increased incidence of any of 74 different diagnostic disease categories was found in the adjuvant cohort, including autoimmune diseases, connective tissue diseases, arthritis, or cancer. As previously concluded [16], the relative lack of toxicity of incomplete Freund's adjuvant in humans illustrates the pitfall of relying on evidence obtained only in rodents.

Aluminum salts

Aluminum salts, first introduced in 1926, are currently the most commonly used adjuvants for vaccines. Although the adjuvant activity of an aluminum salt is often relatively weak, adsorption of multiple antigens to aluminum salts does serve the function of formulating combination vaccines, such as diphtheria-pertussis-tetanus (DPT), DPT-haemophilis influenzae, or hepatitis B-haemophilus influenzae vaccines [28]. In addition, adsorption to alum salt might have the interesting benefit of stabilizing and retarding the degradation of an antigen that degrades with a pH-dependent mechanism [29].

The literature on aluminum adjuvants is quite extensive, and recent research on mechanisms suggests that the adjuvant mechanisms of aluminum and calcium salts may be complex. Excellent reviews have covered the literature and technical characteristics of these adjuvants, and regulatory issues [2, 3, 28, 30]. The commonly used aluminum salt adjuvants include various physical forms of aluminum phosphate, aluminum hydroxide, and aluminum-precipitates of protein [32]. These compounds are currently so widely referred to informally as a group by the term alum that this term has become an accepted generic shorthand in the vaccine field for aluminum salt adjuvants even though the name alum was originally given to potassium aluminum sulfate, a chemical entity that is not actually being used as an adjuvant [3].

Certain vaccines can be created by precipitation of protein with an aluminum salt, but currently the most common method of using aluminum is by simple adsorption, and this is accomplished by mixing of antigen with pre-formed aluminum gel. As noted earlier, even at their best, aluminum salts have only modest adjuvant activity, and they are often inconsistent in their ability to serve as adjuvants for widely used protein antigens, particularly for booster immunization [2, 28]. Different lots or different brands of the same type of aluminum salt can be inconsistent in adsorptive capacity [33]. Despite the deceptive ease of adsorption by bringing protein antigen and aluminum salt together, considerable difficulty in adjuvant reproducibility may be encountered because of the huge complexity of aluminum-protein interactions, an area particularly explored in detailed physicochemical studies by Stanley Hem [34]. This complexity extends not only to the adsorption of antigen during vaccine manufacture, but also due to potential exchange of protein antigen with interstitial proteins and other constituents of interstitial fluids after injection [35–37]. Likewise, it is through the mechanism of cycles of disaggregation and reaggregation of aluminum hydroxide adjuvant that homogeneous mixing of multiple antigens occurs on aluminum adjuvant particles in combination vaccines containing more that one antigen [38].

The adsorptive binding of proteins to aluminum is often due to electrostatic interactions, and adsorption can be strongly affected by the age of

the gel [39], the charge of the gel, the charge of the adsorbed protein, by the physical structure of the aluminum compound, and by the pH and other characteristics of the buffer that is used ([29, 40–42], reviewed in [3]). It is possible to manipulate the electrostatic adsorption in certain cases. Aluminum hydroxide gel is positively charged at pH 7.4 and it adsorbs negatively charged proteins. However, pretreatment of aluminum hydroxide with buffer containing phosphate can lower, or even reverse, the positive charge, even allowing the electrostatic adsorption of cationic protein [43].

Many other mechanisms also play a role in the adsorptive process, including hydrophobic interactions, van der Waals forces, and hydrogen bonding (reviewed in [3]). Recently it was suggested that ligand exchange between the phosphate groups of phospholipids in the hepatitis B surface antigen with the hydroxyl groups of aluminum hydroxide provides a mechanism of high affinity binding [44]. Presumably this mechanism could also account for the binding of phospholipid liposomes containing monophosphoryl lipid A to aluminum hydroxide adjuvant [33, 45].

The dose of aluminum adjuvant is an important consideration. It is important to understand that there may be an optimal level of aluminum for a given vaccine antigen, with decreased immune response at higher levels of aluminum [3]. Although it has been stated that aluminum remains at the site of injection for a long period [3], aluminum salts are known to be removed from the body with time [46]. The acceptable dose of aluminum in adjuvants ranges up to 1.25 mg both by U.S. and by WHO regulations, but currently licensed US vaccines have a range of only 0.125 to 0.85 mg [28]. The latter reference can also be consulted for a list of all licensed US vaccines containing aluminum and the aluminum content of each.

Side-effects of widely used human adjuvants

It is well known that immunostimulators in general, and adjuvants in particular, can sometimes be double-edged swords. Side-effects and toxicities of adjuvants can be widespread and commonplace and the toxicity and reactogenicity of a given adjuvant is often cited as having equal or greater importance than the potency as a criterion to be considered in the selection of an adjuvant [11, 47]. It is the introduction of side-effects or toxicities that are produced in otherwise healthy persons that are the most troubling aspects of vaccine- and adjuvant-induced side-effects, and this is particularly true for pediatric vaccines.

Some local effects, such as local ulceration induced by live Vaccinia organisms that are used for smallpox or the bacillus Calmette-Guérin (BCG) organism that is widely used around the world as a tuberculosis vaccine, and the familiar local redness and swelling at the site of injection induced by aluminum salts [31], are usually tolerated as levels of toxicity

that are generally not so stressful as to preclude the use of the offending materials. It is generally believed that the local inflammation associated with intramuscular injection of aluminum salts is due to the escape of adjuvant along the injection track into the subcutaneous space [12]. Pain after injection and the occurrence of painless nodules after multiple injections of aluminum adjuvanted vaccines are associated with the subcutaneous route of injection [48, 49].

Systemic effects of various adjuvant materials sometimes can be more serious, and these can include: nausea and vomiting that are associated with a small fraction of vaccinated cohorts; pyrogenic effects due to endotoxin that are almost universally found with crude vaccines containing endotoxin; or even uveitis, a rare occurrence that may be associated with certain adjuvants.

Sometimes the adjuvant can even produce a set of symptoms that partly mimics the disease against which the vaccine is directed. Such is the case of orally administered CT or LT, each of which can induce diarrhea similar to that found in cholera or in *E. coli* diarrhea. However, a much more dramatic and dangerous example of toxicity of LT was recently discovered in a licensed Swiss vaccine that was administered intranasally [50, 51]. When LT was included in a liposomal (virosome) vaccine containing hemagglutinin and neuraminidase surface antigens of influenza virus, intranasal administration of the vaccine resulted in a strong association with an increased risk of facial nerve paralysis (Bell's palsy). The increased risk was highest at 31–60 days after vaccination and the toxic effect resulted in withdrawal of the vaccine from the market.

It is known that ganglioside GM1 serves as a high affinity receptor for LT and CT and, although the natural targets for CT and LT are the cells of the intestine, GM1 is widely distributed in many other tissues, including neural tissues [52]. Thus, although the mechanism of the induction of Bell's palsy was not completely clear, it would seem likely that the interaction of LT with GM1 on the olfactory nerve played an important role [51]. This unfortunate neurological toxicity involving the facial nerve was probably uniquely related to the intranasal administration of enterotoxin. Fear of facial nerve toxicity should not have a negative impact on the use of native or modified enterotoxin vaccine adjuvants when given by other routes for immunomodulation and adjuvant effects. Furthermore, there is no reason to suspect that the virosome itself exhibited any toxicity independent of its role as a carrier of LT.

Because of the use of crude antigenic materials contaminated with endotoxin, or even because of the local granulomatous inflammation caused by aluminum salts, the impression has arisen that the function of many adjuvants is mainly to cause an inflammatory reaction and that the toxic effects of the reaction are critical contributors to adjuvant activity. It is true that local inflammation can often enhance the immunogenicity of an antigen, mainly because inflammatory cells contain a large fraction of

phagocytes and lymphocytes that may serve as antigen presenting cells. However, it is not true that toxic effects are necessarily required for adjuvant activity. Quite the opposite: modern adjuvants strive to enhance immunogenicity in a focused manner that minimizes or avoids local adverse reactions or systemic toxicity.

Elimination of the toxicity of Gram-negative bacterial endotoxin, a potent classic adjuvant, while retaining the adjuvant effects provided by the endotoxin, is a dramatic example of the lack of reliance of an adjuvant on toxicity for expression of adjuvant effect. Essentially all of the toxic effects of endotoxin, including pyrogenicity, are due to the lipid A moiety of the lipopolysaccharide that is present on the surface of Gram-negative bacteria [53]. Because of this, the terms endotoxin and lipid A are often used interchangeably, but the toxic effects of purified lipid A are greatly reduced, and the entire adjuvant effect is retained, by utilization of monophosphoryl lipid A instead of the complete diphosphoryl lipid A molecule [54]. Monophosphoryl lipid A still retains some toxicity at high doses, but the residual toxicity, including pyrogenicity, of monophosphoryl lipid A can be completely eliminated by incorporation of monophosphoryl lipid A into liposomes [55]. The complete detoxification of monophosphoryl lipid A by liposomes allows the potential safe use of even higher doses of monophosphoryl lipid A with still higher adjuvant activity, if higher adjuvant activity is indeed required [55, 56].

Lessons from historical adjuvants

Certain types of adjuvant formulations that have been widely used in the past could serve as useful practical starting points for adjuvant selection for many vaccines. Among such materials are included: aluminum salts; lipid A or monophosphoryl lipid A; and a variety of emulsions, including water-in-oil or oil-in-water emulsions. Lipid A is the constituent of Gram-negative bacterial endotoxin that has given particular potency to certain whole cell bacterial vaccines. Each of these adjuvants has advantages and disadvantages involving availability, potency, safety, cost, or ease of manufacture. Improvements on these, such as by the use of liposomes with lipid A, can introduce additional factors or complications.

Improvements on other historical adjuvants that were previously not easily available or not acceptable because of toxicity or other factors, include introduction of cytokines either directly or through the use of DNA coding [57, 58]; highly purified less toxic derivatives of saponin-like materials [19]; and nontoxic analogs of toxins such as analogs of CT and LT. The latter compounds in their native forms as CT or LT are highly toxic as oral mucosal adjuvants, and generally induce diarrhea, but newer derivatives of CT and LT have been introduced that have reduced, or completely lack, toxicity [59]. Even the native forms of CT and LT lack systemic or mucosal

toxicity when applied directly to the skin as adjuvants for induction of transcutaneous immunization [8, 9, 60]. A fascinating discovery has been made that when either native CT or LT is used as an adjuvant with co-administered antigen on the surface of the hydrated skin for transcutaneous immunization, the combined adjuvant formulation can induce both systemic (IgG) immunity [8, 9, 60], cytotoxic T lymphocytes [61], and even mucosal immunity [9, 60, 62] both in animal models and in humans.

Selection of vaccine adjuvants

After a useful and extensive textbook review of the history of adjuvants and of the status of research on current and new generations of vaccine adjuvants, Professor G. J. V. Nossal recorded the following conclusion: "The reader will by now have gathered that the field of adjuvants is not too rich in simplifying or overarching paradigms. It seems likely that empirical research will continue to dominate for some time." [63]. While it is true that this conclusion might seem somewhat grim, it is the same conclusion that could be made of vaccine research in general, and, indeed, for many areas of medical research. However, modern adjuvant research is beginning to identify the hint of useful general paradigms, or at least beginnings of rational explanations of certain adjuvant activities, based on the understanding of dendritic cells as "Nature's adjuvants" that modulate immune responses through activities of pathogen recognition receptors (PRRs), Toll-like receptors (TLRs) and C-type lectins [64].

The field of vaccines is by history and by nature fraught with difficulties, and adjuvants by themselves do not serve as a transforming principle for creating simplicity out of complexity. They also do not necessarily serve as a means for illuminating predictable paths to overcome all of the difficult and diverse obstacles that microorganisms have created against immune attack that allow them to beset humans and other mammals with disease burdens. It is undoubtedly for the reason that combinations of vaccine components are unpredictable with respect to stability, efficacy, and safety, that the U.S. Food and Drug Administration, and many other national regulatory agencies, have not licensed adjuvants as stand-alone products but only as constituents of complete vaccine formulations [65, 66].

Adjuvant mechanisms

Mechanisms of adjuvant expression exist on several levels. At a macro level, a slowly dispersed depot, such as aluminum salt, oil emulsion deposits, or other particulate scaffolding materials may serve either as a slow release mechanism, or as a concentration of antigen and adjuvant that attracts antigen presenting cells that ingest the depot through phagocytosis or endocy-

tosis over a long period of time. At a cellular level, dendritic cells exert activities through the aforementioned PRRs, TLRs, and C-type lectins, leading to steering of the immune response through complicated signaling processes and so-called conversations between cells that are mediated by cytokines, chemokines, and other mediators. Other mechanisms of innate immunity, such as complement activation, can also play an important, or even critical, role in the evolution of the immune response. Mechanisms involving targeting of the antigen to a desired cell, or local delivery of the antigen to a desired location, such as for oral, intranasal, or transcutaneous delivery, can be viewed as a useful adjuvant-like strategy. This also holds true for altering the dose of adjuvant and the timing of vaccination. It seems obvious that if an adjuvant exerts a strong depot effect, it would provide little benefit to provide a booster immunization at the site of the depot before the earlier depot material had been depleted. Discussions of numerous adjuvant mechanisms and available adjuvants have been provided in various reviews [11, 47, 63, 64, 67].

The process of optimization of vaccine adjuvants generally employs multiple mechanisms, which may require putting together groups of adjuvants that exhibit both macro and cellular, and perhaps even targeting, mechanisms. The evolution of understanding of TLRs and PRRs has given some recent useful insights into the rationale of using certain combinations of adjuvants. For example, CpG DNA acts on TLR 9 to trigger both IL-12 and IFN-alpha to induce strong Th1 and cytotoxic T lymphocyte (CTL) responses, while *E. coli* LPS acts on TLR 4 to trigger mainly IL-12 plus a little IFN-alpha and Th1 responses [64]. While it is true that this information could provide a possible rationale for combining adjuvants such as these together, it would be naive to believe that such responses could be easily predicted for any given human vaccine, and it would be naive to believe that true optimization of immune responses could be achieved without providing other mechanisms of targeting or scaffolding.

Commercial adjuvants

There are numerous success stories in the adjuvant field that can allow the selection of useful adjuvants that could be employed for practical success in the creation of important vaccines. A plethora of reviews on adjuvants provides a rich selection of adjuvant candidates that can be examined [11, 12, 47, 63, 67]. A useful compendium of a large number of available vaccine adjuvants can also be accessed electronically on the website of the Division of AIDS at NIAID at the U.S. National Institutes of Health [http://www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf]. But the huge variety of potential adjuvants that are available also presents the further problem that the adjuvant or adjuvant combination for a given vaccine must be additionally selected based on criteria such as ease of manufacture,

cost, potency, and risk/benefit ratio. As Professor Nossal accurately observed: "…each commercial firm interested in the field has simply gone its own way, with little collaboration between the groups." [63]. Many of the best known and best studied adjuvants have been invented through commercial research and are therefore restricted for exclusive use by individual companies. The holding of proprietary adjuvants for their own exclusive use by companies should not be viewed as a serious impediment for many vaccines because the basic reasons for success or failures of most commercially developed adjuvants are well known and it is often not too difficult to create similar generic alternatives.

A more difficult problem is that the use of a particular adjuvant or adjuvant strategy by a company does not necessarily imply that the adjuvant selected by the company is the most effective one that could be employed; indeed, compromises in adjuvant selection are often made by companies based largely on issues of profitability. While this is not necessarily a bad strategy in itself for the company or even the consumer, it might not always be the best initial route for conducting research to attack huge and difficult international diseases, such as HIV/AIDS or malaria, or other important diseases for which potent and safe vaccines have not yet been developed or licensed.

Many of the commercial adjuvants that have been proposed or are being developed appear to have excellent potential for future use, and some have enjoyed success as constituents of licensed vaccines. Although in the U.S. aluminum salts are still the only adjuvants that have been used in any licensed vaccine to date, new types of adjuvants are now in advanced stages of clinical testing for licensing. In several other countries, a variety of novel new aluminum-free commercial adjuvants have already been included in licensed vaccines as described below.

An oil-in-water emulsion (MF59) has been developed and manufactured by Chiron that is used in a licensed influenza vaccine in Europe [17, 18]. Prior to the 2004–2005 influenza season, more than 11 million doses had been administered and the vaccine has proved to be remarkably safe. It is reported to be particularly effective for inducing effective immunity to influenza in elderly vaccinees [17].

A liposome (virosome) containing hepatitis A antigen has been developed by Berna for use as a licensed hepatitis A vaccine in Switzerland [68]. The vaccine appears to be safe, effective, and competitive in the commercial market.

A melanoma vaccine containing an oily dispersion having monophosphoryl lipid A and mycobacterial cell as constituents has been developed by Corixa Corp [69] and licensed in Canada [http://www.corixa.com/default.asp?pid=release_detail&year=2001&ID=19].

While these licensed vaccines serve as examples, and even as inspirations, numerous other adjuvant formulations are working through commercial pipelines toward eventual applications for new-generation vaccines.

A clinical algorithm for selection of vaccine adjuvants

As noted earlier, hundreds of adjuvants and adjuvant formulations have been proposed as immunostimulants for various purposes for vaccines. When combined with a variety of options for route of delivery of the vaccine, the selection process for adjuvant choice presents a maze of complexities. It is therefore not surprising that animal models have often been employed for simplification of the process. Unfortunately, considerable experience with numerous animal models has demonstrated that in many cases conclusions from such models do not accurately predict adjuvant-enhanced immunogenicity or protection against the infection or disease being modeled, and may not even correctly predict the safety profile of the adjuvant [16]. In certain instances animal disease models that might be used for adjuvant selection do not even exist.

One such example that lacked a suitable animal model involved the development of an immunotherapeutic vaccine against prostate cancer. When faced with this latter difficulty, we took it as an opportunity to create an interesting and useful clinical experimental algorithm for rational selection of adjuvants. The challenge in this algorithm had the following parameters. An immunotherapeutic vaccine, utilizing recombinant prostate-specific antigen (PSA), was to be developed for treatment of advanced metastatic prostate cancer under circumstances in which the patients had previously exhausted essentially all available conventional therapeutic options. This situation was particularly challenging because the patients were elderly, extremely ill, and highly immunosuppressed because of cancer. In addition, the difficulty was compounded both by the requirement to overcome immunological tolerance against PSA, a circulating self antigen, and the further requirement to induce cellular immunity, in essence to induce cellular autoimmunity against PSA.

To address this situation, our strategy was to conduct multiple sequential small (5–10 patient) phase I safety and immunogenicity trials with recombinant PSA in which changes and improvements in the adjuvant formulation and route of immunization would be devised based on the results from the previous trial. Liposomes containing encapsulated rPSA and monophosphoryl lipid A (MPL) (Walter Reed liposomes) [33, 45] were selected as a core adjuvant platform technology. These types of liposomes had been shown to induce potent immune responses in previous comparative adjuvant trials in humans for experimental vaccines to malaria [56, 70] and HIV [71].

As summarized in Table 1, six sequential trials were conducted with rationally selected adjuvant formulations [16, 72]. The trials, the rationales for adjuvant formulation, and a summary of the results, are described below. In the first two trials, consisting of intramuscular (i.m.) and intravenous (i.v.) immunization, respectively, with Walter Reed liposomes containing lipid A and rPSA, only 2 of 10 patients exhibited either antibodies

Table 1. Immunization regimens of prostate cancer patients

Trial	Route of immunization	Number of patients*	Dose**	Immunization schedule (days)	Additional adjuvants
1	Intramuscular	6/5	1 ml	0, 30, 60	None
2	Intravenous	6/5	1 ml	0, 30, 60	None
3	Subcutaneous	11/10	1 ml	0, 30, 60	GM-CSF, 125 mg injected subcutaneously into the vaccine site on the day of immunization and for 4 days thereafter
4	Intradermal	5/5	0.25 ml to 4 sites	0, 1, 2, 15, 30	Cyclophosphamide, 300 mg/m^2 intravenously on days –3 and 27; BCG, 0.01 ml of a 1:10 dilution injected into the vaccine sites on Study Days 0, 15, and 30
5	Intramuscular	9/8	0.9 ml	0, 30, 60	Vaccine emulsified in oil-in-water (o/w) emulsion in light mineral oil
6	Subcutaneous	5/5	1 ml	0, 30, 60	Cyclophosphamide, 300 mg/m^2 intravenously 3 days before each immunization; GM-CSF, 125 mg injected subcutaneously into the vaccine site on the day of immunization and for 4 days thereafter; IL-2 self-administered subcutaneously by the patient in a dose of 3 million IU/m^2 daily on days 7–13, 37–43, and 67–73

* Number of patients available for evaluation for safety/immunologic responses.
** Liposomes containing recombinant PSA, 100 mg/ml and lipid A, 200 mg/ml.

or DTH responses to PSA. The i.v. route was selected in order to increase delivery of the vaccine to the lymphocytes in the spleen, but this strategy failed to induce any detectable immune response. We concluded that although the liposomes by themselves had previously proved quite potent, they still did not have sufficient potency for this unique challenge.

The next two trials were conducted simultaneously. In trial 3, GM-CSF was added to the core liposome formulation containing rPSA and MPL because it had been shown to enhance maturation of dendritic cells and macrophages [73] and to increase antigen presentation of protein and peptide-based vaccines [74–77]. GM-CSF was injected subcutaneously at the vaccine injection site on the day of immunization and four days after immunization. This strategy finally worked – immune responses were measured in 100% of the patients. Nine of 10 had antibodies to PSA and 7 of 10,

Table 2. Immune responses following immunization with liposomal PSA containing lipid A

Trial	Route of immunization	Additional adjuvants	Antibodies to PSA*	Lymphocyte proliferation**	DTH*** (induration)
1	Intramuscular	None	1/5	0/5	1/5
2	Intravenous	None	0/5	0/5	0/5
3	Subcutaneous	GM-CSF	9/10	4/10	7/10
4	Intradermal	Cyclophosphamide, BCG	4/5	3/5	2/5
5	Intramuscular	Liposomal oil-in-water emulsion	7/8	6/8	7/8
6	Subcutaneous	Cyclophosphamide, GM-CSF, IL-2	3/5	5/5	2/5

* Number of patients/total number patients immunized having serum antibody titers to PSA >6,400.

** Number of patients/total number patients immunized having positive proliferative responses to PSA. Data are normalized to the response obtained from PBMC obtained prior to immunization.

*** Number of patients/total number patients immunized having induration >10 mm following skin challenge with PSA.

including the patient who did not develop antibodies to PSA, had >10 mm induration upon challenge with PSA in a skin test (Tab. 2) [78]. Four patients also had lymphocyte proliferative responses to PSA.

In trial 4, cyclophosphamide was administered 3 days prior to immunization with the liposome formulation because cyclophosphamide had been reported to augment delayed-type hypersensitivity responses to antigens [79, 80]. BCG was used because it had been shown to be effective for prophylaxis and therapy of cancer [81, 82]. Four of 5 patients developed antibodies to PSA following immunization, but only 2 patients had induration after skin test challenge with PSA. One patient had no demonstrable immune responses following vaccination. Three patients also had lymphocyte proliferation responses to PSA.

Trial 5 used a novel liposome-stabilized oil-in-water liposomal emulsion [83, 84]. The liposomes containing lipid A and rPSA were used as a stabilizer for emulsifying light mineral oil. During the emulsification process some of the liposomes were degraded and coated the oil droplets, and this had the effect of stabilizing the emulsion. Seven of 8 patients immunized with the emulsion developed antibodies to PSA. Seven of 8 patients had induration after a skin test with PSA [78] and 6 of 8 were positive for PSA-induced lymphocyte proliferation (Tab. 2).

The last formulation tested (Trial 6) consisted of cyclophosphamide pretreatment prior to immunization, injection of GM-CSF at the injection site and interleukin 2 (IL-2) subcutaneous administration every day for one week starting a week after vaccination. IL-2 was included because it has been shown to enhance immune response in patients undergoing vaccine

therapy by expanding cells with antigen-induced high affinity receptors [85] and up-regulating T-cell cytokines [86]. Three of 5 patients had antibodies to PSA and 2 of those patients had induration reactions following skin challenge with PSA. All of the patients had PSA-induced lymphocyte proliferative responses (Tab. 2).

The PBMC from patients immunized in trials 3 and 5 were further analyzed for PSA-induced interferon-γ (INF-γ) secretion by ELISPOT analysis [87]. CD4 and/or CD8 PSA-responding cells were observed in samples from both trials. In addition, vaccination decreased the percentage of zeta-negative $CD3^+$ cells routinely observed in cancer patients and increased the percentage of IL-10-secreting cells [78].

Based on the immune responses, the formulations that utilized GM-CSF or emulsion were judged to be superior to the other formulations. The liposome-stabilized emulsion was further tested in colon cancer patients using liposome-encapsulated recombinant KSA, a formulation also manufactured at Walter Reed [88]. Antibody responses and cellular immune responses to KSA, as measured by lymphocyte proliferation, DTH and INF-γ ELISPOT responses, were induced in this latter study following immunization with the emulsion. By using our clinical algorithm strategy of employing successive small clinical trials, we were able to identify a unique new liposomal oil-in-water emulsion as a potent new vaccine adjuvant formulation.

From this successful experience we believe that a similar clinical research algorithm could be used with almost any antigen as a cost-effective method to optimize the immunogenicity of the antigen with competing formulations of rationally selected adjuvant candidates and routes of delivery. In those instances involving chronic or difficult diseases, such as HIV/AIDS, malaria or cancer, where successful creation of a protective vaccine has encountered considerable barriers Draconian measures may be required involving extremely potent adjuvants. The clinical research algorithm described above may therefore have general utility as a means to provide rational guidance for adjuvant selection.

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Vaccination in the context of immunological immaturity

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Introduction

Neonates, infants, and young children are at higher risk of infection and are more susceptible to serious consequences of infections than adults. This susceptibility results from limitations of both innate and adaptive (antigen-specific) immunity. All major lineages of hematopoietic cells that are part of the immune system are present in the human by the beginning of the second trimester, but their state of differentiation and their functional capacity may limit the immune response. In this chapter, we review general information about phenotypic and functional studies of antigen-presenting cells (APCs), T cells and B cells in the human neonate, infant, and young child, including responses to specific infectious agents and vaccines. To illustrate recent insights into the acquisition of antigen-specific immunity in the context of the developing immune system after the neonatal period, we summarize our recent investigations of immune responses in infants and young children with post-natally acquired human cytomegalovirus (CMV) infection and infants given live attenuated measles vaccine at six, nine or twelve months of age.

Antigen presentation and dendritic cells

CD8 T cells usually recognize foreign peptide antigens presented by major histocompatibility complex (MHC) class I molecules, which consist of HLA-A, -B, and -C in humans. MHC class I expression is almost ubiquitous. Most MHC class I-bound peptides are 8–10 amino acids in length and are derived from proteins recently synthesized *de novo* within host cells [1]. CD4 T cells usually recognize peptide antigens presented by MHC class II molecules, which consist of HLA-DR, -DP, and -DQ in humans. MHC class II antigen is usually expressed on professional APCs, which include den-

dritic cells, mononuclear phagocytes, and B cells. Most MHC class II peptides are from 14–18 amino acids in length, and mostly derived from phagocytosis or endocytosis of soluble or membrane-bound protein. MHC class I and class II molecule expression by human fetal tissues is evident by 12 weeks of gestation [2, 3]. MHC class I expression by neonatal lymphocytes is lower than on adult cells [4], but the significance of this difference is not known. Importantly, relatively normal levels of basal MHC surface expression do not exclude subtle deficiencies in antigen presentation in the neonate and young infant, particularly under conditions that more stringently test APC function. Infection with herpesviruses, such as herpes simplex virus (HSV) that can inhibit peptide loading of MHC class I, is an example [5, 6]. The amount of MHC class II expression per cell on neonatal monocytes or B cells is similar or greater than that of adult cells [4], but fewer neonatal monocytes may express HLA-DR [7]. Whether this decreased expression is functionally significant and continues beyond the neonatal period is unknown.

Myeloid dendritic cells (DCs), which are also known as DC1-lineage cells, play an essential role in antigen presentation to naïve CD4 and CD8 T cells and the initiation of the primary immune response. DC1s in uninflamed tissues are referred to as immature because they express only moderate levels of MHC class I and class II molecules. Immature DC1s are also found in the blood, and are likely to be in transit to the tissues from sites of production in the bone marrow. After exposure of immature DC1s to inflammatory stimuli, such as cytokines or microbial pathogen-derived ligands that bind their Toll-like receptors (TLRs) [8], further antigen uptake ceases. As part of maturation, antigenic peptides derived from previously internalized particles are displayed on cell surfaces in the groove of MHC class I and II molecules. Increased surface expression of the CCR7 chemokine receptor during maturation facilitates DC migration *via* the lymphatics into T-cell-rich areas of secondary lymphoid organs that express the CCR7 ligand chemokines, CCL19 and CCL21 [9].

Mature DC1s express high levels of peptide-MHC complexes and molecules that act as costimulatory signals for T-cell activation, such as CD80 (B7-1) and CD86 (B7-2). These features make them highly efficient APCs for the activation of naïve CD4 and CD8 T cells [10]. DCs are unique among APCs in being able to present antigenic peptides on MHC class I molecules by cross-presentation, in which extracellular proteins that are taken up as large particles (phagocytosis), small particles (macropinocytosis), or in soluble form (micropinocytosis) are transferred from endocytic vesicles to the cytoplasm and then onto MHC class I molecules [11]. Cross-presentation is essential for the activation of naïve CD8 T cells by antigenic peptides from pathogens that do not directly infect DCs. DCs also influence the quality of the T-cell response by directing the differentiation of naive CD4 T cells into Th1 (capable of producing IFN-γ but not IL-4, IL-5, or IL-13) or Th2 (capable of producing IL-4, IL-5, or IL-13 but not IFN-γ) effec-

tor T cells. For example, the production of IL-12 by DCs skews differentiation towards the Th1 pathway [12–14].

DC2-lineage cells, also known as plasmacytoid DCs, are a distinct DC subtype that have an important role in immunity by secretion of high levels of cytokines, particularly type I interferon (IFN-αs and IFN-β), in response to certain pathogens or pathogen-derived products. The secretion of type I interferon may not only provide a systemic anti-viral effect but also enhance the ability of myeloid DCs to present exogenous acquired antigens to CD8 T cells by cross-presentation [15]. Most DC2-lineage cells circulate as pre-DC2 precursors that lack cytoplasmic protrusions characteristic of immature or mature DC1s and have a limited capacity to present antigen. Although mature DC2s can present antigen to T cells, at least *in vitro*, their importance for antigen presentation to T cells *in vivo* is less clear.

Because most T-cell responses in the neonate and young infant are to neoantigens, any quantitative or qualitative differences in DC function would be expected to limit T-cell responses. Epidermal Langerhans cells and dermal DCs are found in the fetal skin by 16 weeks of gestation [16], and immature DC1 lineage cells are found in the interstitium of solid organs by this age [17]. Cells with the features of pre-DC2s are found in fetal lymph nodes between 19–21 weeks of gestation [18]; they have an immature phenotype and are not recent emigrants from inflamed tissues. In normal adults, circulating DCs represent about 0.5% of circulating blood mononuclear cells and consist usually of immature DC1s and pre-DC2s. In the neonatal circulation, pre-DC2s with a HLA-DR^{mid}CD11c-CD33-CD123$^{mid\text{-}hi}$ surface phenotype and lacking markers for other cell lineages (Lin^{-}) predominate in early infancy. They constitute about 75% of circulating DCs and ~0.75% of total blood mononuclear cells [19–22]. The remaining 25% of circulating DCs have an HLA-DR^{hi}CD11c^{+}CD33^{+}CD123^{lo} surface phenotype that is similar to that of circulating adult DC1s, except that CD83 expression is absent [20]. The number of circulating DC2-lineage cells appears to decline with increasing post-natal age, whereas the number of circulating DC1s does not. The predominance of DC2 lineage cells in the neonatal and infant circulation may reflect their high rate of colonization of newly formed lymphoid tissue, which undergoes rapid expansion at this age.

Circulating DC1s and pre-DC2s from adults and neonates are similar in their basal expression of HLA-DR and costimulatory molecules that are important in T-cell activation, such as CD40, which binds to CD40-ligand on T cells, and CD80 and CD86, which bind to CD28 on T cells [19, 23]. Stimulation of neonatal DC1s with lipopolysaccharide (LPS), a ligand for TLR4, and poly I:C, which is a surrogate for double-stranded RNA, a ligand for TLR3, increases their expression of HLA-DR and CD86 to adult DC1 levels. However, these stimuli are less effective for increasing CD40 and CD80 on neonatal DC1s [23]. Stimulation of neonatal and adult pre-DC2s with unmethylated CpG DNA (a ligand for TLR9 that is a compo-

nent of DNA from bacteria or herpesviruses) increases their expression of HLA-DR similarly; however, CD40, CD80 and CD86 are increased to a lesser extent for neonatal pre-DC2s. In contrast to adult DC1s, neonatal DC1s do not upregulate expression of HLA-DR, CD80, and CD86 in response to pertussis toxin [24].

Circulating DCs from cord blood can allogeneically stimulate cord blood T cells *in vitro* [20, 25, 26], but their efficiency has not been directly compared with adult DCs. Virtually all of this allostimulatory activity is mediated by DC1s rather than pre-DC2s [20], which raises the possibility that neonatal DC1 function may be normal on a per cell basis. A *caveat* is that the activation of allogeneic T cells does not require uptake, processing and presentation of exogenous antigens, and is not as stringent a test of DC1 function as activation of foreign antigen-specific T cells.

The influence of DCs on Th1 *versus* Th2 phenotype is dependent on the type of DC and the particular activation conditions [27–30]. Antigen presentation by pre-DC2 favors Th2 differentiation unless these cells have been activated by viruses or unmethylated CpG DNA that cause them to release type I IFN or IL-12 that drive Th1 polarization [28]. The predominance of pre-DC2s in the fetus, neonate, and young infant might limit their response to intracellular pathogens, if this applies to the tissues. Neonatal DCs also appear to produce less type I IFN and IL-12 compared to DCs from adults, as inferred from studies using peripheral blood mononuclear cells (PBMCs) or whole blood, and assuming that DCs are the dominant source of these cytokines. For example, type I IFN production or the frequency of IFN-α-producing cells in response to HSV [31], parainfluenzae virus [32], and unmethylated DNA [19] was lower in assays using neonatal blood compared to adult blood. In the case of the response to unmethylated DNA, this decreased production is accounted for by differences between neonatal and adult pre-DC2 cells [19]. Neonatal blood cells also produce less IFN-α than adult cells in response to poly I:C, a stimulus that most likely acts on DC1s *via* TLR3 [23]. In aggregate, these studies suggest that neonatal DC1 and DC2 lineage cells produce ~10–30% as much type I IFN as analogous adult cells.

Circulating DC1s appear to be the major source of IL-12 in assays using PBMCs or whole blood. LPS induces less IL-12 production by neonatal blood mononuclear cells than by adult cells. Cord blood monocyte-derived DCs, which serve as a model of DC1 cells, also have a low capacity to produce IL-12 in response to LPS, engagement of CD40, or treatment with poly I:C [33]. Since IL-12 plays a key role in directing naïve T cells towards Th1 differentiation, this might account for limitations in adaptive immunity to LPS-containing pathogens, such as *Salmonella*. In contrast, neonatal and adult blood mononuclear cells stimulated with *Staphylococcus aureus*, other Gram-positive and Gram-negative bacterial cells, or meningococcal outer membrane proteins produce equivalent amounts of IL-12, suggesting that decreased IL-12 production is relatively stimulus-specific [34–37].

T cells

T cells mainly originate in the thymus and play a central role in antigen-specific immunity by directly mediating cellular immune responses and by facilitating antigen-specific humoral immune responses by B cells. Most T cells in the blood and peripheral lymphoid organs express antigen-specific T-cell receptors (TCR) that are heterodimeric molecules composed of TCR-α and TCR-β chains [38], with the amino-terminal portion of these chains being variable and involved in antigen recognition. The highly variable nature of this portion of the TCR is generated by intrathymic TCR gene rearrangement of variable (V), diversity (D), and joining (J) segments for TCR-β and V and J segments for TCR-α. Additional diversity of the TCR is provided by the random addition of nucleotides (referred to as N-nucleotides) to V, D, and J segments during the joining process by the enzyme terminal deoxytransferase (TdT). The TCR on the cell surface is invariably associated with the nonpolymorphic complex of CD3 proteins, which serve as docking sites for intracellular tyrosine kinases that transduce activation signals to the interior of the cell after the TCR has been engaged by antigen [39].

T-cell production, TCR repertoire, and surface phenotype

The production of peripheral T cells is established by the second trimester of pregnancy, with the concentration of circulating CD4 and CD8 T cells found at birth being substantially higher than in adults [40]. The αβ-TCR repertoire of cord blood T cells that is expressed on the cell surface has a diversity of TCR-β usage and a distribution of complementarity determining region 3 (CDR3) lengths that are similar to those of antigenically-naïve T cells in adults and infants. The CDR3 region is the most important source of TCR diversity and is formed by the junction of V segments with D and J segments. Thus, the functional pre-immune repertoire is fully formed by birth [41–43].

Circulating T cells of the healthy neonate typically lack markers for memory T cells, such as the CD45R0 isoform, β1 integrins (e.g., VLA-4), and, in the case of CD8 T cells, killer inhibitory receptors [44] and CD11b [45, 46]. This is consistent with a predominance of an antigenically-naïve population due to limited prenatal exposure to foreign antigens. The proportion of circulating T cells with a memory/effector phenotype, i.e., $CD45R0^{hi}$ surface expression, increases gradually with post-natal age [40, 47], presumably due to cumulative post-natal antigenic exposure.

CD38, an ectoenzyme that generates cyclic ADP-ribose, is expressed on most thymocytes, and some activated peripheral blood T cells and B cells, plasma cells, and DCs. Unlike adult naïve T cells, virtually all peripheral fetal and neonatal T cells express the CD38 molecule [48-51], suggesting

that peripheral T cells in the fetus and neonate may represent a thymocyte-like immature transitional population. Neonatal CD4 T cells lose expression of CD38 after *in vitro* culture with IL-7 for 10 days [52], which suggests that loss of CD38 may occur with further maturation independently of engagement of the αβ-TCR/CD3 complex.

T-cell proliferation and IL-2 production

Most early studies comparing neonatal and adult T-cell activation used unfractionated T cells or unpurified circulating mononuclear cells. However, memory T cells typically have more robust signaling and activation-induced gene expression than naïve T cells [53–57], and naïve T cells may be more dependent on co-stimulation *via* engagement of CD28 [58]. This discussion will focus on more recent studies in which neonatal T cells, either unfractionated or purified based on high expression of the CD45RA marker, were compared with naïve T cells from adults since these are more likely to be informative for *bona fide* ontogenetically-related differences in activation or effector function.

Compared to adult naïve CD4 T cells, neonatal naïve CD4 T cells produce less IL-2 mRNA and express fewer high-affinity IL-2 receptors in response to stimulation with anti-CD2 monoclonal antibody (mAb) [59–61]. These differences were abrogated when phorbol ester, which bypasses proximal signaling pathways by activating Ras proteins and protein kinase C, was included [59]. This suggests a relatively inefficient generation of proximal signals after T-cell activation. Similarly, the production of IL-2 by neonatal naïve CD4 T cells is reduced compared to adult naïve ($CD45RA^{hi}$) CD4 T cells after allogeneic stimulation with adult monocyte-derived DCs (D. Lewis, unpublished data), again arguing that neonatal cells may be intrinsically limited in their ability to be physiologically activated for IL-2 production.

The ability of activated T cells to efficiently divide in response to IL-2 depends on the expression of the high-affinity IL-2 receptor, which consists of CD25 (IL-2 receptor alpha chain), the beta chain (shared with the IL-15 receptor), and the γc cytokine receptor (a chain shared with multiple other cytokine receptors). Neonatal T cells express similar or higher amounts of CD25 after stimulation with anti-CD3 mAb [62]. Although basal expression of the γc cytokine receptor is lower by neonatal T cells than by either adult naïve ($CD45RA^{hi}$) or memory ($CD45R0^{hi}$) T cells [63], the importance of this finding is unclear since activated neonatal T cells appear to proliferate in response to exogenous IL-2 as well as or better than adult T cells [62].

Two *in vitro* studies suggest that neonatal T cells may be less able to differentiate into effector cells in response to neoantigen. One study using newborns who were not infected with CMV found that the frequency of

neonatal T cells proliferating to whole inactivated CMV antigen was significantly less than that of adult T cells from uninfected adults [64]. A pitfall of this study is that it used a complex antigen preparation, and one to which the T cells of adults might have previously been primed by infections other than CMV. Another study [65] found decreased antigen-specific T-cell proliferation and IL-2 production by PBMCs incubated with a protein neoantigen (keyhole limpet hemocyanin). Although these results require confirmation, they are consistent with the more limited ability of neonatal naïve CD4 T cells to produce IL-2 in response to allogeneic DCs than adult naïve cells (D. Lewis, unpublished data).

Production of other cytokines and chemokines

Many studies (reviewed in [66]), suggest that the CD4 T-cell subset or unfractionated T cells of the neonate have a reduced capacity to produce a wide range of cytokines, including IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and GM-CSF, compared to adult cells. However, since memory/effector T cells have a markedly greater capacity to produce these cytokines than naïve T cells, in most instances the apparent deficit of the neonatal T cells is accounted for by the lack of a circulating memory/effector cell population. However, we have found that highly purified naïve CD4 T cells from neonates have a substantially reduced capacity to produce IFN-γ *in vitro* compared to adult naïve CD4 T cells following 24–48 hours of stimulation with the same pool of monocyte-derived DCs from multiple unrelated blood donors [67]. This was demonstrable using cell culture supernatants as well as single-cell assays of CD4 T cells using intracellular cytokine staining [67]. The mechanism for this decreased expression of IFN-γ appears to involve decreased expression of CD40-ligand by neonatal T cells as well as decreased IL-12 production by the DCs, as both of these factors are important in this system for early Th1 differentiation (D. Lewis, unpublished data).

This strongly suggests that the capacity of neonatal naïve CD4 T cells to produce IFN-γ is intrinsically more limited, even when a potent, physiological APC population is used for antigen presentation. Reduced expression of IFN-γ by neonatal CD4 T cells is associated with greater methylation of DNA at a few sites in the IFN-γ gene locus in neonatal T cells compared to adult naïve T cells [68, 69]. Whether neonatal and adult naïve CD4 T cells differ at multiple other sites in their methylation of the IFN-γ gene or in other epigenetic modifications that may influence IFN-γ gene expression remains to be explored.

Highly purified neonatal naïve CD4 T cells have a limited increase in the intracellular concentration of free calcium ($[Ca^{2+}]_i$) following anti-CD3 mAb stimulation compared to identically treated adult naïve cells [70]. Although the mechanism for this reduced calcium response remains to be

defined, it is likely to contribute not only to reduced production of cytokines, such as IFN-γ, but also of important cell surface molecules, such as CD40-ligand, since an elevated $[Ca^{2+}]_i$ is important for the *de novo* transcription of these genes. Neonatal naïve CD4 T cells also express reduced levels of certain transcription factors, such as NFATc2 [71, 72], which require increases in $[Ca^{2+}]_i$ for their function and that are a key factor for inducing multiple cytokine genes, including IFN-γ, and CD40-ligand. These intrinsic T-cell deficiencies, in conjunction with immature DC function may account for delayed production of IFN-γ by antigen-specific CD4 T cells following infection in early infancy.

Although cytokine production by neonatal CD8 T cells has not been as well characterized as for the CD4 T-cell subset, neonatal naïve CD8 T cells produce significantly more of the Th2 cytokine, IL-13, than adult naïve cells after stimulation with anti-CD3 and anti-CD28 mAbs and exogenous IL-2 [73]. Whether this unusual cytokine profile, which needs to be confirmed, applies to antigen-specific immune responses, such as to viral pathogens, is unclear.

Expression of TNF ligand family members

CD40-ligand (CD154), a member of the TNF ligand family, is expressed on the cell surface in high amounts by activated but not resting CD4 T cells. CD40-ligand engages CD40, a molecule expressed by APCs, including DCs, mononuclear phagocytes, and B cells. Cell-cell interactions involving CD40-ligand and CD40 are essential for many events in adaptive immunity, including the generation of memory CD4 Th1 cells, memory B cells, and, as discussed below, most immunoglobulin isotype switching [74]. In most studies, neonatal CD4 T cells, including purified naïve cells, have a much more limited capacity to express CD40-ligand and accumulate CD40-ligand mRNA than adult naïve CD4 T cells after pharmacological activation with calcium ionophore and phorbol ester [75–78]. Thus, this may represent a true developmental limitation in activation-induced gene expression. Human $CD4^{hi}CD8^{-}$ thymocytes, the immediate precursors of naïve CD4 T cells, also have a low capacity to express CD40-ligand [78, 79], suggesting that neonatal peripheral CD4 T cells have delayed post-thymic maturation of this function. However, use of a pharmacological stimulus might not accurately mimic physiological T-cell activation. Although we [70] and others [80] observed similar relative reductions in CD40-ligand surface expression by purified neonatal naïve CD4 T cells compared to adult naïve CD4 T cells using more physiological stimuli that engage the αβ-TCR/CD3 complex, this reduction was not observed in studies from two other laboratories [81, 82]. This suggests that the particular *in vitro* conditions employed may influence the outcome of the assay.

There are also conflicting results as to whether CD40-ligand expression by neonatal T cells is decreased after allogeneic stimulation. One study using irradiated adult monocyte-derived DCs as allogeneic stimulators, found that CD40-ligand expression by neonatal T cells was similar to that of adult T cells after 5 days of culture [83]. In contrast, we observed substantially lower levels of CD40-ligand expression by purified naïve CD4 T cells than adult naïve CD4 T cells after 24–48 hours of such allogeneic stimulation [67]. We favor a model in which limited initial expression of CD40-ligand by neonatal naïve CD4 T cells can be overcome with continued priming. Thus, the differentiation of neonatal naïve CD4 T cells into Th1 effector cells by CD40-ligand- and IL-12-dependent processes may both be limited during the early stages of T-cell differentiation. Although limitations in CD40-ligand production could contribute to decreased antigen-specific immunity in the neonate and young infant [74], little is known about the adequacy of CD40-ligand expression following activation of naïve CD4 T cells by antigen *in vivo*, which primarily occurs in the T-cell-rich regions of peripheral lymphoid tissue.

Fas ligand (CD95L), another member of the TNF ligand family, is important in inducing apoptotic cell death on target cells that express Fas on the surface, including lymphocytes, myeloid cells, and hepatocytes. Neonatal T cells have decreased Fas ligand expression after anti-CD3 and anti-CD28 mAb stimulation compared to unfractionated adult T cells [80], but it is unclear whether there is also a decrease relative to adult naïve cells.

Co-stimulation and anergy

Neonatal T cells produce IL-2 and proliferate as well as do adult T cells when optimal sources of CD28 co-stimulation are provided (e.g., transfectants expressing the CD28 ligands CD80 or CD86, or CD28 mAbs) [60, 61, 84]. However, neonatal CD4 T cells have a greater tendency than adult $CD45RA^{hi}$ T cells to become anergic and functionally unresponsive when they are activated *in vitro via* the αβ-TCR/CD3 complex without concurrent optimal co-stimulation [85]. This anergic tendency may be relevant since newborns with toxic shock syndrome-like exanthematous disease, in which Vβ2-bearing T cells are markedly expanded *in vivo* by the Staphylococcal exotoxin, TSST-1, have a greater fraction of anergic Vβ2-bearing T cells than do adults with TSST-1-mediated disease [86].

Chemokine receptor expression

The differential expression of chemokine receptors by T cells is important in their selective trafficking to sites of antigen presentation *versus* inflamed tissues [87]. CCR7 expression by naïve T cells allows these cells to recircu-

late between the blood and uninflamed lymphoid organs. Naïve T cells of the adult express CCR1, CCR7, and CXCR4 on the cell surface, and have low to undetectable levels of CCR5. The role served by CCR1 and CXCR4 expression on naïve T cells is unclear, and CCR1 may be non-functional in this cell type [88]. Infant and adult naïve T cells have a similar phenotype, except that infant cells lack CCR1 surface expression and, unlike adult naïve T cells, they do not increase CXCR3 expression and decrease CCR7 expression after activation *via* anti-CD3 and anti-CD28 mAb [89, 90]. The increased expression of CXCR3 and decreased expression of CCR7 is important for allowing T cells to enter inflamed tissues that express chemokine ligands for CXCR3. The CCR7 expressed on neonatal T cells is functional and mediates chemotaxis of these cells in response to cognate chemokines [91]. Together, this suggests that activated neonatal T cells may be limited in their capacity to traffic to non-lymphoid tissue sites of inflammation and, instead, may continue to recirculate between the blood and peripheral lymphoid organs.

Memory T-cell subsets

Memory T cells are heterogeneous in their expression of CCR7 [92]. The $CCR7^{hi}$ population (putative central memory cells) appears to preferentially recirculate between the secondary lymph nodes and blood, have limited effector function, and may serve as a reservoir for the generation of additional memory cells. The $CCR7^{lo}$ cell population (putative effector memory cells), is enriched in memory cells that can rapidly induce effector functions, such as IFN-γ or IL-4 production or cytotoxin expression. The $CCR7^{lo}$ cell subset is also enriched in the expression of other chemokine receptors, which facilitates their preferential entry into inflamed or infected tissues [87]. Central memory cells may be intermediates between naïve T cells and effector memory T cells [93], although this remains highly controversial [94].

Our study of CMV-specific CD4 T-cell responses showed that the memory CD4 T-cell subset of infants and young children had a significantly higher ratio of central memory to effector memory CD4 T cells than adults [66]. This difference may reflect reduced activity of the IL-12/IL-23-dependent Th1 pathway, since effector memory CD4 T cells are markedly reduced in IL-12Rβ1 deficiency, which ablates IL-12 and IL-23 signaling [95]. Circulating monocytes in infants also have a reduced capacity to produce IL-12 compared to older children and adults that could contribute to this mechanism [96]. Although the numbers of circulating effector memory CD4 T cells may be reduced, this population appears to be functionally normal. For example, the frequency of effector memory CD4 T cells that produced IFN-γ and the amount of IFN-γ produced per cell in response to the *Staphylococcus aureus* toxin SEB was similar in the blood of infants and young children compared to adults [66].

T-cell cytotoxicity

Effector and memory CD8 T cells kill more efficiently than antigenically-naïve T cells after stimulation with lectins or anti-CD3 mAb [97] or after allogeneic sensitization [98-100]. Thus, the early reports of deficiency of neonatal CTL activity after *in vitro* activation or priming (reviewed in [66]) can be explained by the absence among neonatal CD8 T cells of effector and memory cells, as identified by their expression of CD45R0 and/or their lack of CD27 and CD28 [101]. Until recently, the capacity of fetal T cells to mediate cytotoxicity received little scrutiny. One recent study documented robust fetal effector CD8 T-cell responses, including clonal expansion, IFN-γ production, and perforin expression, in response to congenital CMV infection [102]. A robust fetal CD8 T-cell response also appears to occur in cases of congenital infection with the parasite *Trypanosoma cruzi*, the agent of Chagas disease [103]. Thus, the capacity to generate a functional CD8 T-cell effector population *in vivo* is established *in utero*, at least under conditions of chronic stimulation. These studies, as well as our studies of the CD8 T-cell response to primary CMV infection in infants and young children, which are discussed below, suggest that CD8 T-cell responses to persistent viral infection are similar to those of adults. However, it is unclear whether this applies to CD8 T-cell responses that occur more acutely, e.g., within one week, such as in response to respiratory viral infections.

Antigen-specific CD4 T-cell immunity

Infants between 6–12 months of age have lower IL-2 production in response to tetanus toxoid than older children and adults [104]. This suggests that either antigen-specific memory CD4 T-cell generation or function is decreased during early infancy. Whether this reflects limitations in antigen processing, T-cell activation and co-stimulation, or proliferation and differentiation remains unclear.

In contrast to inactivated vaccine antigens, BCG vaccination at birth *versus* 2 months or 4 months of age was equally effective in inducing CD4 T-cell proliferative and IFN-γ responses to purified protein derivative (PPD), extracellular *M. tuberculosis* antigens, and a *M. tuberculosis* intracellular extract [33, 105]. The responses were robust not only at 2 months following immunization but also at one year of age, and there was no skewing towards Th2 cytokine production [105], even by PPD-specific CD4 T-cell clones [33]. Thus, early post-natal administration of BCG vaccine does not result in decreased vaccine-specific Th1 responses, tolerance, or Th2 skewing. How these responses compare to older children and adult vaccinees is not known. Early BCG vaccination may also influence antigen-specific responses to unrelated vaccine antigens. BCG given at birth increased Th1- and Th2-specific responses and antibody titers to hepatitis B surface

antigen (HBsAg) given simultaneously [106]. BCG vaccine given at birth did not enhance the Th1 response to tetanus toxoid given at 2 months of age, but did increase the Th2 response (IL-13 production). It is likely that BCG vaccination may accelerate DC maturation so that these cells can augment either Th1 or Th2 responses.

The T-cell-specific response to oral poliovirus vaccine (OPV), another live vaccine, suggests a decreased Th1 response. Neonates given OPV at birth, 1, 2, and 3 months of age, have lower OPV-specific CD4 T-cell proliferation, IFN-γ production, and the number of IFN-γ-positive cells than previously immunized (but not recently reimmunized) adults [107]. In contrast, their antibody titers were higher than those of adults, suggesting that CD4 T-cell help for B cells is not impaired. OPV may be less effective at inducing a Th1 response than BCG in neonates and young infants because of its limited replication, site of inoculation or ability to stimulate APC in a manner conducive to Th1 immunity, relative to BCG, which induces persistent infection in the recipient.

Although neonates and young infants have been suggested to have skewing of CD4 T-cell responses towards a Th2 cytokine profile, this may be an oversimplification. For example, the tetanus toxoid-specific response following vaccination indicates that both Th1 (IFN-γ) and Th2 (IL-5 and IL-13) memory responses occur, particularly following the third vaccine dose at 6 months of age [108]. The tetanus toxoid-specific Th1 response may decrease transiently by 12 months of age, while Th2 responses are not affected [108].

Antigen-specific CD8 T-cell immunity

CD8 T-cell responses to CMV infection acquired *in utero* [102] or during infancy and early childhood [109] are robust. Cytotoxic responses to human immunodeficiency virus (HIV) in perinatally infected infants suggest that CD8 T cells capable of mediating cytotoxicity have undergone clonal expansion *in vivo* as early as 4 months of age [110]. However, their cytotoxicity may be reduced and delayed in appearance compared to adults [111]. There is also decreased HIV-specific CD8 T-cell production of IFN-γ by young infants after perinatal HIV infection [112], and an inability to generate HIV-specific cytotoxic T cells following highly-active anti-retroviral therapy [113]. When evaluated beyond infancy, cytolytic activity directed to HIV envelope proteins was commonly detected, but cytolytic activity directed against gag or pol proteins was rarely detected [114], suggesting that the TCR repertoire of cytotoxic CD8 T cells was less diverse than in adults.

HIV-1 infection may inhibit antigen-specific immunity by depleting circulating DCs [115], impairing antigen presentation [116], decreasing the output of naïve T cells by the thymus [117], and promoting T-cell apoptosis [118]. In addition, maintenance of HIV-specific CD8 T cells with effector

function depends on HIV-specific CD4 T cells, which may be selectively and severely impaired by the virus. Regardless of the precise mechanism, the suppressive effects of HIV-1 on cytotoxic responses may be relatively specific for HIV-1, since HIV-infected infants who lack HIV-specific cytotoxic T cells may maintain cytolytic T cells against Epstein Barr virus (EBV) and CMV [112, 113]. A surprising and poorly characterized effect of HIV-1 infection is to inhibit the responses of HIV-exposed but uninfected infants born to HIV-infected mothers [117, 119].

An early study found that respiratory syncytial virus (RSV)-specific cytotoxicity was more pronounced and frequent in infants 6-24 months of age than in younger infants [120]. Murine studies indicate that RSV infection suppresses CD8 T-cell-mediated effector activity (IFN-γ production and cytolytic activity) and that only transient memory CD8 T-cell responses occur following infection [121]. Longitudinal studies of CD8 T-cell immunity to RSV in children and adults following primary and secondary infection will be of interest to determine if this immunoevasive mechanism applies to humans.

In summary, T-cell function in the fetus, neonate, and likely the young infant, is impaired compared to adults. T-cell participation in T-cell help for B-cell differentiation is diminished. Selectively decreased expression of activation-dependent proteins by fetal and neonatal T cells, such as cytokines and CD40-ligand, may contribute to these deficits. The repertoire of αβ-TCR is probably adequate except in early fetal gestation. Following fetal or neonatal infection, the acquisition of CD4 T-cell antigen-specific responses may be typically delayed. *In vitro* studies suggest that deficiencies of DC function and intrinsic limitations in the activation and differentiation of antigenically-naïve CD4 T cells into memory/effector cells may be contributory, although the function of tissue-associated DCs is unknown. In contrast to diminished CD4 T-cell function, CD8 T-cell-mediated cytotoxicity and cytokine production in response to strong chronic stimuli, such as congenital CMV infection, appears to be intact in the fetus and neonate.

B cells

Mature B cells are identified by their expression of surface immunoglobulin (sIg). Immunoglobulin (Ig), which is synonymous with antibody, is a heterotetrameric protein consisting of two identical heavy chains and two identical light chains linked by disulfide bonds [122]. Like the TCR, the amino terminal portion of the antibody chains is highly variable as a consequence of the assembly of V, D, and J gene segments (Ig heavy chain) or V and J segments (Ig light chain). However, antibody molecules are distinct from the αβ-TCR in that they typically recognize antigens based on their three-dimensional structure, such as those found on intact proteins or on

non-protein molecules, such as complex carbohydrates. The B-cell antigen receptor (BCR) is also distinct from the TCR in that Ig variants are generated in mature B cells by the process of somatic hypermutation, in which germinal center B cells accumulate apparently random point mutations within existing V, D, and J segments. These variants undergo a selection process favoring B cells that bear sIg with high affinity for antigen. As for T cells, B cells are activated to proliferate and differentiate after engagement of their sIg antigen-specific receptor. sIg is invariably associated with nonpolymorphic membrane proteins, Ig-α (CD79a) and Ig-β (CD79b), which are structural and functional homologues of the CD3 complex proteins, and are involved in the intracellular transmission of activating signals to the interior of the cell.

For B cells to be activated effectively and to produce antibody against protein antigens requires help from T cells in most cases. This help is in the form of soluble cytokines, such as IL-4 and IL-21 [123], and of cell surface–associated signals, such as CD40-ligand, which is transiently expressed on the surface of activated CD4 T cells. The engagement of CD40 on the B cell is also instrumental in inducing B cells to undergo Ig isotype switching, for example, from IgM to IgE, in which the constant region at the carboxy-terminus of the Ig heavy chain gene is replaced with another isotype-specific segment but the antigen-combining site at the amino-terminus is preserved. In cases in which the antigen has multiple and identical surface determinants (e.g., complex polysaccharides or certain viral proteins with repetitive motifs) and multiple sIg are cross linked, antigen binding alone may be sufficient to induce B-cell activation without cognate (direct cell-cell interaction) help from T cells. In this case, other signals derived from non–T cells or T cells, such as cytokines, or from microorganisms, such as bacterial lipoproteins or pathogen-derived DNA containing unmethylated CpG motifs, may enhance antibody responses [124]. Isotype switching, however, is much more limited in this context. As for T cells, B cells receive additional regulatory signals from the engagement of surface molecules other than the BCR that act as either co-stimulatory or inhibitory molecules. A B-cell co-stimulatory molecular complex consisting of CD19, CD21, and CD81 [125] binds the CD3d fragment of the C3 complement component. B-cell activation is suppressed when a surface receptor for the Fc (fragment crystallizable) portion of IgG, FcγRIIB, is engaged concurrently with the sIg by antigen-IgG complexes [126], and, as discussed below, this may be a mechanism by which maternal antibody inhibits the B-cell response of infants.

B-cell production, Ig repertoire, and surface phenotype

The proportion of B cells in the spleen, blood, and bone marrow is similar to that in the adult by 22 weeks of gestational age [127, 128]. Mature B cells

circulate at higher concentration during the second and third trimesters than at birth, and concentrations decline further by adulthood [50, 129]. The Ig repertoire of peripheral B cells of the neonate and young infant is similar to that of the adult based on V and D segment usage. Certain segments (e.g., DH7-27) [130] may be over-represented in the neonate and certain V segments may be lacking [131, 132], but this is unlikely to limit the humoral immune response of the neonate and young infant. Other V segments, such as VH3, are present at a greater frequency in the pre-immune Ig repertoire of the neonate [132, 133], which allow antibody molecules to bind protein A of *Staphylococcus aureus*, perhaps providing some intrinsic protection during the perinatal period.

The CDR3 region is the most hypervariable portion of Igs, and is at the center of the antigen-binding pocket of the antibody [134]. Thus, reduced CDR3 diversity could limit the efficiency of the antibody response. The CDR3 region of the Ig heavy chain gene remains relatively short until the beginning of the third trimester due to a lack of activity of the TdT enzyme. Subsequently, the CDR3 length gradually increases in length so that by birth it is similar to that of adult B cells [130, 135–137]. A complete lack of nucleotide additions by TdT would be predicted to result in antibodies with combining sites that are relatively flat and potentially inefficient at combining with antigen [138], but the importance of shortened CDR3 regions by themselves in limiting antibody responses is doubtful: gene knockout mice lacking TdT produce normal antibody responses following immunization or infection [139]. A combination of a relative lack of TdT and limitations in V and D usage could limit the ability of the fetal B cells to recognize a full spectrum of foreign antigens, particularly prior to mid-gestation, but this is unlikely to occur in term neonates and young infants.

The B cells of neonates and young infants have increased surface levels of IgM compared to adult B cells [140, 141]. In flow cytometric studies in which non-specific binding is carefully excluded, $<1\%$ of circulating B cells of the neonate express surface IgG or IgA [142], indicating that isotype switching prior to birth is rare. Consistent with this finding, true germinal centers in the spleen and lymph nodes are absent during fetal life, but appear during the first months after postnatal antigenic stimulation [143].

A high frequency of circulating and lymphoid tissue-associated fetal and neonatal B cells express CD5, indicating that most belong to the B-1a subset [144–147]. These circulating CD5+ B cells are prominent at birth [40] and gradually decline with increasing postnatal age [145, 148, 149]. Like adult B-1a cells, newborn B-1a cells express IgM antibodies that are polyreactive, including with self antigens, such as DNA [145, 146, 150, 151]. Neonatal B cells also differ from adult B cells in expressing CD10 (CALLA), which typically is limited in the adult to immature B-lineage cells of the bone marrow. Thus, similar to the finding of a thymocyte marker, CD38, on neonatal T cells, CD10 expression by some neonatal B cells raises the possibility of a maturational arrest, although $CD10^+$ B cells are

functionally mature based on their ability to undergo isotype switching. The small fraction of $CD10^+$ B cells found at birth gradually declines during infancy [152]. Neonatal B cells also have reduced expression of CD21, which is involved in co-stimulation of B cells by complement [153], and of the FcγRII receptor (CD32) [140, 154], which may render them less subject to the inhibitory effect of antigen/antibody complexes. Reduced expression by neonatal $CD5^-$ B cells of the adhesion molecules CD11a, CD44, CD54 (ICAM-1), and L-selectin has also been reported [155, 156], which might reduce their ability to traffic to tissues from the circulation compared to adult B cells.

T-cell-dependent Ig production and isotype switching

Most studies have found that neonatal or fetal B cells have similar capacity to adult naïve B cells to undergo isotype switching and produce IgM, the four subclasses of IgG, IgA, and IgE, if they are optimally activated using exogenous cytokines (e.g., IL-4 or IL-10) and by CD40 engagement (reviewed in [66]). However, neonatal B cells may produce substantially less IgA than do adult naïve B cells in the presence of adult T cells stimulated by anti-CD3 mAb (as a source of CD40-ligand) and IL-10 [157], suggesting intrinsic limitations of B-cell function, particularly when T-cell help may be limited. This decrease in isotype switching is not attributable to decreased neonatal B-cell activation or proliferation, since these cells proliferate like adult B cells after engagement of CD40 and/or surface IgM [141].

In systems in which neonatal B cells are dependent on neonatal T cells for a source of CD40-ligand, Ig production and isotype switching are reduced compared to when adult T cells are used [78]. However, as discussed above, it remains controversial whether CD40-ligand production by neonatal T cells limits immune responses *in vivo*. Whether decreased neonatal DC function also contributes to diminished B-cell responses has not been determined, but an increasing body of literature suggests that direct interactions between B cells and DCs may be important for regulation of B-cell immunity.

B-cell response to T-independent antigens

T-independent (TI)-type I antigens are those which bind to B cells and directly activate them *in vitro* to produce antibody without T cells or exogenous cytokines. In the human, one such TI-1 antigen is fixed *Brucella abortus*, which is likely to utilize TLRs for B-cell activation [158]. TI-type II antigens are mostly polysaccharides with multiple identical subunits, and certain proteins that contain multiple determinants of identical or similar anti-

genic specificity. Responses to these antigens are enhanced *in vitro* and *in vivo* by a variety of cytokines, including IL-6, IL-12, IFN-γ, and GM-CSF [159, 160–162]. NK cells, T cells, or macrophages may produce these cytokines *in vivo*. TI-type II responses are also enhanced by bacterially derived LPS, lipoproteins, porin proteins, or unmethylated CpG DNA [161, 163, 164]. This enhancement probably occurs primarily by engagement of TLRs on B cells [124]. The response to TI-type II antigens is characterized by the lack of B-cell memory or somatic hypermutation and is largely restricted to the IgM and IgG2 isotypes [153].

Antibody production by human neonatal B cells to a TI-type I antigen *in vitro* (*Brucella abortus*) is only modestly reduced [165]. This appears to be due to a decreased ability of antigen-activated B cells to proliferate rather than a decreased precursor frequency of antigen-specific clones [165]. The response to TI-type II antigens is the last to appear chronologically. This is likely to account, at least in part, for the susceptibility of the neonate and young infant to infection with encapsulated bacteria, e.g., such as group B streptococci [166, 167] and, as discussed in detail below, the poor response to bacterially derived polysaccharide antigens in vaccines until after 2 years of age. These poor antibody responses are associated with the lack of circulating memory ($CD27^{hi}$) B cells that express IgM and have not undergone isotype switching in children less than 2 years of age [168]. The reduction or absence of these IgM memory B cells in adult patients after splenectomy suggests that they may depend on the spleen microenvironment for their generation or long-term survival.

Whether the decreased response to TI-type II antigens of early childhood is an intrinsic B-cell immaturity and/or is due to decreased function of other cells such as APCs of the spleen, remains unclear. Decreased CD21 expression by infant B cells has been proposed as a possible mechanism for limitations in the TI-type II response. CD19 and CD21 together constitute the type 2 complement receptor, which serves to transduce B-cell activating signals after its engagement by C3 complement components and promote cell proliferation, and murine experiments support an important role for this receptor for antibody responses to pathogens, such as *Streptococcus pneumoniae* [169]. Studies using human splenic tissue suggest that TI–type II antigens activate complement and bind C3 and subsequently localize to the marginal zone splenic B cells expressing type 2 complement receptors [153, 170]. The acquisition of a capacity to respond to bacterial polysaccharides at approximately 2–3 years of age correlates with the appearance of B cells expressing CD21 in the marginal zone region of the spleen [171]. Although recent studies have not found decreased expression of CD21 by circulating neonatal B cells [140, 172], it remains possible that B cells that preferentially traffic to the marginal zone could have such decreased expression. Mechanisms for reduced responses to polysaccharides that involve APC populations of the splenic marginal zone have also not been excluded. Neonatal B cells appear to have intact pathways of activation *via*

TLR9 [156], suggesting that this does not account for the poor response to TI-type II antigens at this age.

B-cell response to T-dependent antigens

Most proteins are T-dependent antigens requiring cognate T-cell/B-cell interaction for production of antibodies (other than small amounts of IgM). Conjugation of polysaccharide antigens with protein carriers also results in responses to the polysaccharide moiety that is T-dependent. The antibody response to T-dependent antigens is characterized by the generation of memory B cells with somatically-mutated, high-affinity Ig and the potential for isotype switching. The production of CD40-ligand by CD4 T cells and the engagement of CD40 on B cells are critical for these B-cell responses.

The capacity of humans to respond to T-dependent antigens is well established at birth, and is only modestly reduced compared to the response of the adult. There are many potential mechanisms for this modest reduction including: decreased DC interactions or function with CD4 T cells or B cells; limitations in CD4 T-cell activation and expansion into a T helper-effector cell population; impaired cognate interactions between CD4 T cells and B cells; an intrinsic B-cell defect; or a combination of these factors. Another possibility is that T-dependent antigens preferentially upregulate CD22 on neonatal B cells compared to adult B cells, and which may raise the threshold for B-cell activation on neonatal compared to adult B cells [173]. Most studies of the neonatal immune response to T-dependent antigens have not evaluated antibody affinity, a reflection of somatic mutation, or isotype expression. Although such responses might be limited early in the immune response, e.g., because of decreased CD40-ligand expression by CD4 T cells [70, 75, 76, 78, 174], this has not been documented. Decreased CD40-ligand expression by antigen-specific T cells would also be expected to result in reduced memory B-cell development, but this has not been documented for B cells of the neonate and young infant responding to T-dependent antigens.

Antibody responses to vaccines

Immunization starting in the neonatal period, even within the first 48 hours after birth, usually elicits a protective response to T-dependent protein antigens, including tetanus and diphtheria toxoids [175], OPV [176], hepatitis B surface antigen (HBsAg) [177], and the experimental bacteriophage ϕX174 vaccine [178]. The response to some vaccines may be less vigorous in the neonate than in older children or adults. For example, this has occurred in the primary antibody response to recombinant HBsAg in term neonates

lacking maternally-derived HBsAg antibody, compared to unimmunized older infants, children, and adults [177, 179]. Anti-HBsAg titers achieved in infants after the second and third doses are similar to those of older children, indicating that early immunization does not result in tolerance [177]. If initial immunization is delayed until one month of age, the antibody response to primary HBsAg vaccination is increased and nearly equivalent to older children, suggesting that the developmental limitations responsible for reduced antibody responses are transient [177, 180]. Similarly, although two-week-old infants immunized with a single dose of diphtheria or tetanus toxoid had delayed production of specific antibody compared to older infants, by two months of age their response was similar to that of six-month-old infants [181]. Isotype switching from IgM to IgG may also be delayed following neonatal vaccination for some vaccines, such as *Salmonella* H antigen [167] but not all antigens, e.g., bacteriophage ϕX174 [178]. Immunization of infants of HIV-infected mothers starting at birth with recombinant HIV-1 gp120 vaccine in MF59 adjuvant also elicited high antibody titers without evidence of tolerance [182].

In contrast to most inactivated vaccines, newborns given whole cell pertussis vaccination may not only have a poor initial antibody response, but a subsequently reduced antibody response to certain antigenic components, such as pertussis toxin, compared to infants first immunized at 1 month of age or older [183-185]. This suggests tolerance induction, which is relatively vaccine-specific.

The response to purified polysaccharide antigens, such as from *Haemophilus influenzae* type b or group B streptococci, is absent or severely blunted when these vaccines are given in the neonatal period. The response to some polysaccharide antigens, such as from *Neisseriae meningitidis* type A, can be demonstrated by 3 months of age, but the response to vaccination with polysaccharides from *H. influenzae* type b, *Neisseriae meningitidis* type C, or most pneumococcal serotypes, is poor until approximately 18–24 months [186]. As discussed above, this inability to respond to polysaccharides and other TI-type II antigens is not clearly understood, but does not appear to be due to limitations in expression of an appropriate antibody repertoire [187].

Coupling of the *H. influenzae* type b polysaccharide or other polysaccharides to a protein carrier converts a TI-type II antigen to a T-dependent antigen [188]. This is accompanied by an enhanced magnitude and higher avidity antibody response on subsequent boosting, presumably resulting from T-dependent memory B-cell generation and somatic hypermutation. Conjugation of *H. influenzae* type b capsular polysaccharide covalently to a protein carrier renders it immunogenic in infants as young as 2 months of age, and primes for an enhanced antibody response to unconjugated vaccine given at 12 months of age. Since this is an age when the response to the unconjugated vaccine is usually poor, the conjugate vaccine has induced polysaccharide-specific B-cell memory [189]. Conjugation of *H. influenzae*

type b polysaccharide to tetanus or diphtheria toxoid does not change the repertoire of the anti-polysaccharide antibodies produced from that observed using vaccination with the free polysaccharide [187, 189]. Vaccination with protein-capsular polysaccharide conjugate vaccines containing polysaccharides of *Streptococcus pneumoniae* (types 4, 6B, 9V, 14, 18C, 19F, 23F) [190–192] and *N. meningitidis* (types A and C) [193] is immunogenic in infants as young as 2 months of age, and primes them for subsequent memory responses. Thus, the neonatal and young infant response to conjugate vaccines now mimics their response to other T-dependent antigens.

The administration of a single dose of *H. influenzae* type b polysaccharide-tetanus toxoid conjugate to term neonates as early as a few days of age may enhance the antibody response to unconjugated *H. influenzae* type b polysaccharide vaccine at 4 months of age [194]. However, this enhanced response is weak and does not occur when the neonate is primed with tetanus toxoid followed by immunization with conjugate vaccine at two months of age [195].

Inhibition of post-natal antibody responses by maternal antibodies

Maternal antibody can inhibit the production by the newborn or young infant of antibodies of the same specificity. The degree of inhibition varies with the maternal antibody titer, and the type and amount of antigen. For example, maternal antibody has been reported to markedly inhibit the response to measles and rubella vaccine, but not to mumps vaccine [196]. Inhibition of the response to live attenuated viral vaccines may result in part from reduced replication of vaccine virus in the recipient. Maternal antibodies may also inhibit the response to non-replicating "dead" vaccines such as whole cellular pertussis vaccine [185], diphtheria toxoids [197], *Salmonella* flagellar antigen [167] and inactivated poliovirus vaccine [198]. This inhibition may be due to masking of immunogenic epitopes by maternal antibody so that these cannot be bound to sIg on antigen-specific B cells. Another mechanism may involve the formation of antigen/maternal IgG antibody complexes that inhibit activation of B cells by simultaneous engagement of sIg and the inhibitory FcγRII receptor by the IgG component of the complex. Maternal antibody may also lead to more rapid clearance of vaccine antigen and decreased immunogenicity. Despite these multiple potential inhibitor mechanisms, for certain antigens, such as HBsAg, neither maternal antibodies nor hyperimmune IgG administration has a substantial inhibitory effect on the newborn immune response to HBsAg vaccination.

In summary, the inability of the neonate and young infant to produce antibodies in response to polysaccharides, particularly bacterial capsular polysaccharides, limits resistance to bacterial pathogens for up to 2 years of

age. The basis for this defect remains unclear, but may reflect an intrinsic limitation of B-cell function or a deficiency in the anatomical microenvironment or APC function required for B cells to become activated and differentiate into plasma cells. In contrast, neonatal immunization with most T-dependent vaccines results in intact IgM responses and only slightly diminished IgG responses without evidence of tolerance, except possibly for pertussis vaccination. These modest differences between neonates and older infants in the magnitude of the antibody response to protein neoantigens rapidly resolves following birth, and may reflect transient limitations in T-cell function, such as reduced CD40-ligand production, as well as intrinsic limitations of B-cell maturation and function.

T-cell immunity to cytomegalovirus (CMV) infection in infants and young children

Early studies by our group at Stanford and at the University of Washington in Seattle showed that infection of neonates with HSV resulted in antigen-specific proliferation and cytokine (IL-2, IFN-γ, and TNF-α) production by CD4 T cells. However, these responses were reduced in magnitude and substantially delayed compared to adults with primary HSV infection [199, 200]. The postnatal age at which the kinetics of this response becomes similar to that of adults is not known, although clinically HSV rarely causes central nervous system or disseminated disease after the neonatal period.

Many infants and young children become infected with CMV by exposure to maternal CMV in cervical secretions or breast milk, or by close contact with infected children, e.g., in day care [201]. In recent studies, we compared the CD4 and CD8 T-cell immune responses to CMV in young children with post-natally acquired CMV infection to investigate possible developmental limitations in the immune response to this common herpesvirus. Although infected children are almost always asymptomatic, primary CMV infection of neonates and infants usually results in continuous or frequent viral shedding into the urine and saliva for up to several years [201, 202]. In contrast, limited studies have indicated that adults with primary CMV infection stop continual viral shedding by 9–12 months after acquisition, and have only infrequent recurrences of shedding thereafter [203, 204]. The reason for age-related differences in the duration of CMV shedding after primary viral acquisition is unknown but T-cell immunity appears to be important to maintain CMV in a latent state [205, 206]. Studies of murine CMV indicate that CD4 T cells producing IFN-γ, i.e., Th1 cells, are important for the control of CMV replication and shedding from epithelial sites, such as the salivary glands [207–209]. Our hypothesis was that differences in the duration of CMV shedding and the quantity of virus detected in urine by PCR assay between young children and adults would correlate with quantitative or qualitative differences in the T-cell-mediated

immune response, as measured by flow cytometric analysis of whole blood specimens stimulated with CMV or CMV peptides.

Analyses of CMV-specific CD4 T-cell responses

In these studies, frequencies of CMV-specific CD4 T cells were assessed in 13 asymptomatic young children with primary CMV infection of approximately 8–29 months duration and 14 asymptomatic CMV-seropositive adults. Following the approach of Waldrop and Picker [210], we analyzed the frequency of IFN-γ^+ cells using intracellular cytokine staining, and only considered cells that were both IFN-γ^+ and CD69$^+$ to be true-positives for IFN-γ expression. In agreement with previous studies by others [210–212], adults had discrete subsets of CD4 and CD8 T cells that co-expressed IFN-γ and CD69, an activation-dependent protein, in response to either whole CMV antigen (CMV) or a mixture of CMV pp65 (UL83) peptides (pp65) [66]. A control antigen (mock-infected and lysed fibroblasts) and a control peptide pool derived from carcinoembryonic antigen (CEA) amino acid sequence, a self-antigen, resulted in a minimal response (<0.07% IFN-γ^+ cells) similar to incubation of cells without additive.

In contrast, young children had a markedly and significantly ($P<0.05$ by the two-tailed, unpaired Student's t-test) lower frequency of circulating CMV-specific CD4 T cells that produced IFN-γ compared to adults. Children also had a markedly and significantly reduced frequency of CD4 T cells that produced IFN-γ in response to the pp65 peptide mixture. Since activation of pp65-reactive CD4 or CD8 T cells by the peptide mixture requires only peptide binding to MHC surface molecules and not antigen uptake and processing by APCs [211, 213], these results indicated that the decreased CMV-specific CD4 T-cell response of children was not due to impaired APC function during the ex vivo stimulation. These significant decreases of CMV-specific CD4 T cells for young children were likely to apply to the absolute number of these cells. Although total lymphocyte counts per microliter were not determined as part of this study, we reanalyzed the data, assuming that the young children had on average a 2.0-fold greater absolute number of CD4 T cells than the adults, the largest difference reported in the literature [214], all of the differences that were significant for CMV-specific and pp65-specific CD4 T-cell frequency remained significant when expressed as cells/μl of blood.

CMV-specific CD4 T cells producing IFN-γ have been detected as early as 30 days following viral exposure in adults with primary CMV infection as a result of renal transplantation, and who receive potent immunosuppressive drugs to limit transplant rejection [215, 216]. However, it remained plausible that the reduced IFN-γ response by CD4 T cells of healthy CMV-infected young children reflected their shorter duration of CMV infection compared to the healthy adult group with presumed chronic infection. To

exclude this, we compared the CMV-specific Th1 immune responses of 4 healthy adults with primary CMV infection of an estimated 12 to 15 months of duration with those of children for whom the estimated period of CMV infection was 12 to 29 months, i.e., it was equal to or greater than that of the adults. The CMV- and pp65-specific Th1 immune responses of adults with recent primary infection were similar to those of the adult chronic group and, again, were significantly greater than those of the children with primary infection of a similar duration ($P<0.05$). Thus, healthy young children had a markedly limited ability to mount a CMV-specific Th1 immune response for a year or more following viral acquisition, while this lag in anti-viral immunity was not observed in adults.

To assess whether the reduced CMV-specific Th1 response of young children reflected a generally limited ability to mount Th1 immune responses to antigens, we examined the phenotype of CMV-specific CD4 T cells that produced IFN-γ in adults and children. Previous studies have reported that IFN-γ production by adult CD4 T cells in response to protein antigens [217–219], including CMV [210] or SEB [95], is largely confined tothe $CD45RA^{lo}CD45R0^{hi}$ memory subset. We first verified that this applied to CMV-specific and SEB-induced IFN-γ production by CD4 T cells from adults with presumed chronic CMV infection or adults and children with documented recent primary infection.

Next, since memory CD4 T cells expressing low levels of CCR7 have been reported to be highly enriched in IFN-γ production after engagement of CD3 and CD28 [93, 95], we analyzed the relationship between CCR7 expression and Th1 responses for CMV-specific and SEB-activated memory CD4 T cells: we found that among adult and child memory CD4 T cells, more than 90% of cells that produced IFN-γ in response to either stimulation with CMV antigen, the pp65 peptide mixture, or SEB were $CCR7^{lo}$. Similar results were also observed for CD4 T cells from adults with documented recent primary CMV infection. These results suggested that Th1 responses after postnatal antigenic exposure, or at least to CMV-derived antigens, are largely restricted to a $CCR7^{lo}$ memory CD4 T-cell subset. The finding of this restriction of SEB-induced Th1 responses to the $CCR7^{lo}$ subset of memory CD4 T cells in children and adults suggests that this is likely to be a general feature of the CD4 T-cell response to most antigens.

Since the CMV-specific Th1 response was limited to the $CCR7^{lo}$ memory CD4 T-cell subset, we considered whether the reduced CMV-specific Th1 responses of children reflected a general tendency to accumulate fewer $CCR7^{lo}$ cells during memory CD4 T-cell generation. In fact, the fraction of memory CD4 T cells that were $CCR7^{lo}$ was modestly but significantly lower in children with documented recent primary CMV infection (about 50–60% of the adult value) compared to adults with chronic CMV infection [66]. A trend was also observed in a comparison of children and adults with primary CMV infection of similar duration. Although additional studies of other antigen-specific immune responses are needed, these results suggest

that infants and young children may have a tendency to accumulate a lower fraction of CCR7^{lo} cells as part of memory CD4 T-cell responses than adults, and that this may contribute to reduced Th1 immunity to pathogens, such as CMV.

The ability of CCR7^{lo} memory CD4 T cells to produce IFN-γ was further compared between children and adults with documented primary CMV infection of similar duration. The CCR7^{lo} subset of memory CD4 T cells of children had a markedly and significantly ($P < 0.05$) lower frequency of IFN-γ-producing cells in response to whole CMV antigen or the pp65 peptide mixture than the same adult cell population. This was in agreement with the earlier results that analyzed all CD4 T cells or memory CD4 T cells. In contrast, after SEB stimulation, the child and adult CCR7^{lo} subsets of memory CD4 T cells contained a similar frequency of cells producing IFN-γ, and similar amounts of IFN-γ produced per cell, based on mean fluorescent intensity measurements. Thus, the decreased CMV-specific Th1 response by CCR7^{lo} cells in young children was relatively selective in that it did not apply to all Th1 immune responses.

As observed for IFN-γ, the frequency of CMV-specific CD4 T cells expressing IL-2 or CD40-ligand was substantially and significantly ($P<0.05$) lower for children compared to adults with similar duration of primary CMV infection. Interestingly, in contrast to IFN-γ expression, IL-2 and CD40-ligand were expressed by a small but detectable fraction of CMV-specific adult CCR7^{hi} memory CD4 T cells, although a greater fraction was found in the CCR7^{lo} subset. This is consistent with IL-2 and CD40 ligand expression not requiring Th1 differentiation for their effective expression. Nevertheless, the expression of IL-2 and CD40-ligand by the CD4 T cells of the children was essentially undetectable for either the CCR7^{lo} or the CCR7^{hi} subsets, indicating it generally applied to memory cell responses, and these differences were significant when compared with adult cells ($P < 0.05$). CMV-specific CD4 T cells from children and adults were also analyzed for production of IL-4, IL-10, and IL-13 using intracellular cytokine staining, but we were unable to detect cells expressing these cytokines in any samples. This argues against developmentally related skewing of CMV-specific CD4 T-cell responses to produce Th2 or regulatory cytokines rather than Th1 cytokines.

Analysis of CMV-specific CD8 T-cell responses

In parallel with the CD4 T-cell studies, we analyzed the frequencies of CMV-specific CD8 T cells in 17 children, ages 17–41 months (median 24 months), who were shedding CMV in urine, and in 23 CMV-seropositive adults, who were presumed to have chronic infection for years to decades. Since CMV-specific CD8 T-cell frequencies may vary in chronically-infected adults [212], adult subjects were tested twice, at a one-year interval. The

mean frequency of CMV-specific IFN-γ^+ CD8 T cells was 0.4% ± 0.1 (SE) in children tested at a median of 13 months after onset of CMV shedding in urine. The mean frequency in the adults was 0.7% ± 0.2 at the initial testing and 0.5% ± 0.1 after 12 months. Comparing CMV-specific CD8 T-cell responses in children compared to adults at the first time point suggested a difference ($P=0.04$), but not compared to the same adults tested at 12 months ($P=0.2$).

As for CD4 T cells, CD8 T-cell stimulation was also done with the peptide mixture for the CMV pp65 protein, which is known to be a major target for CMV-specific CD8-T cells [213, 220]. The mean IFN-γ^+ CD8 T-cell frequency after pp65 stimulation was 0.3% ± 0.1 in children compared to 0.4% ± 0.1 in adults at initial testing ($P=0.09$) and 0.5% ± 0.1 in adults at 12 months ($P=0.01$). Again, the CMV-specific CD8 T-cell frequencies in chronically-infected adults indicated a difference from children at one time point, but this was not confirmed by the second comparison.

CMV-specific CD8 T-cell frequencies were also determined in five HLA-A*0201 children and five HLA-A*0201 chronically-infected adults using a pp65-HLA-A*0201 tetramer [221, 222]. These experiments were done to assess CD8 T-cell numbers independently of the capacity to produce IFN-γ or other effector functions, which might be reduced in children. The range of tetramer positive CD8 T cells was 0.3–4.6% and did not differ between children and adults (P = 0.5). Perforin expression by tetramer positive cells was also measured in order to determine whether CD8 T-cells had the functional capacity to mediate cytotoxicity, and a similar percentage of these cells were found in children (34%) as in adults (41%) (P = 0.4). Since inadequate differentiation of virus-specific CD8-T cells could result in abnormal effector functions, the pp65-HLA-A*0201 tetramers were used for simultaneous assessment of CD28, which is downregulated during the later stages of CD8 T-cell differentiation [101]. All tetramer$^+$ CD8 T cells from one actively infected child were CD28$^-$ with most cells expressing perforin, as was observed in two chronically-infected adults. Thus, we found no evidence of a limitation in cytotoxic effector function or differentiation of CD8 T cells in infants and young children following primary CMV infection.

Relationship of CMV-specific CD4 and CD8 T-cell frequencies to viral shedding

Previous studies have documented prolonged urinary and salivary shedding of CMV in young children acquiring this infection in the daycare setting [202, 223]. This applied to the young children in our study based on measurements of CMV DNA levels in urine using a real-time PCR assay and viral culture of urine. All children were positive by both assays, indicating that viral shedding in the urine persisted for at least 12–29 months

after acquisition of CMV. In contrast, CMV was not detected by either assay for urine from any adult with recent primary infection. To determine if this result was typical for adults with primary CMV infection, we also retrospectively analyzed data on a group of 30 healthy women with documented primary CMV infection. We found that 90% of these subjects ceased urinary shedding by 6 months after acquisition of virus. Together, these findings demonstrate a striking inverse correlation between viral shedding and a delay in the CD4 but not the CD8 T-cell CMV-specific immune response.

Since chronically-infected adults were likely to have been infected for many years, we compared four adults and 12 children with a similar duration of CMV infection. Three adults had CMV seroconversion within an estimated 12–16-month period but were not shedding virus when evaluated. One adult had primary infection documented by onset of CMV shedding in urine 12 months previously; this adult had a viral load of 1×10^4 copies/ml and stopped shedding after eight months. The 12 children had an estimated duration of infection of 12-28 months, with a mean of 16 months. All 12 children were actively shedding, with a mean of 1×10^4 copies/ml (range: 1×10^3–1×10^5 copies/ml) at the time their CD4 and CD8 T cells were analyzed. Despite these differences in shedding, the frequency of CMV-specific or pp65-specific CD8 T cells detected by CMV antigen stimulation was not different for actively infected children compared to adults. Further, CMV or pp65-specific CD8 T-cell frequencies did not significantly correlate with reduced CMV viral load in urine, which averaged $1\times10^{4.6}$ genome copies/ml (range: 1×10^2–-10^5 genome copies/ml). Thus, high viral loads were detected in some children with the highest CMV-specific CD8 T-cell frequencies, and conversely, low viral loads were observed in some children with the fewest CMV-specific CD8 T cells. In addition, CMV viral load was not related to duration of shedding in these children. Thus, these findings suggest that the circulating levels of CMV-specific CD8 T cells are not predictive of the control of urinary shedding following primary CMV infection in children.

Summary of CMV-specific studies

Previous studies have shown that healthy infants and young children who acquire CMV infection after birth have prolonged viral urinary and salivary shedding compared to adults [201, 202, 205, 224], but the mechanism of this prolonged viral shedding has until now remained unclear. Our results demonstrate a selective limitation in Th1 immunity in young children with postnatally acquired primary CMV infection. This is paralleled by a striking inverse correlation between viral shedding and the CMV-specific Th1 response. The importance of Th1 responses in control of CMV shedding is strongly supported by studies of murine CMV, in which CD4 T cells pro-

ducing IFN-γ but not CD8 T cells were found to be critical for limiting CMV replication in acinar epithelial cells and consequent salivary shedding [207–209]. This is likely to apply to other epithelial sites of shedding, such as the kidney. In contrast, control of CMV replication at other tissue sites, such as the lung, depends critically on CD8 T cells but not CD4 Th1 cells [209]. We speculate that the limited consequence of reduced Th1 responses in these healthy children (i.e., asymptomatic but prolonged CMV shedding) is due to the fact that children develop reduced but detectable CMV-specific Th1 immunity and have robust CD8 T-cell responses. However, we cannot exclude that CMV-specific CD8 T cells of young children and infants might be limited in their ability to traffic to sites of viral replication, such as renal epithelium.

The decreased CMV-specific Th1 response of young children was mainly attributable to a lack of CMV-reactive Th1 cells among the $CCR7^{lo}$ memory CD4 T-cell population. Thus, our results indicate that antigen-specific Th1 immune responses *in vivo* in humans, or at least those that are involved in anti-viral immunity, may be largely mediated cells that are $CCR7^{lo}$. Importantly, $CCR7^{lo}$ memory CD4 T cells were present in substantial numbers in young children and had a similar capacity to produce IFN-γ after SEB simulation. SEB activates T cells based on Vβ segment usage by the αβ-TCR rather than peptide antigenic specificity [225]. Therefore, these IFN-γ^{+} T cells induced by SEB are likely to have undergone clonal expansion and Th1 differentiation in response to a diverse group of peptide antigens. This suggests that the reduced CMV-specific CD4 T-cell IFN-γ production of children is likely to be selective, and not due to a general inability of their $CCR7^{lo}$ memory CD4 T cells to mediate Th1 effector function, or related to an inability to generate Th1 cells from naïve precursors. Further studies evaluating CCR7 restriction of the responses to other antigens will be of interest in young children compared to adults.

We observed that the proportion of circulating memory CD4 T cells that were $CCR7^{lo}$ in young children was approximately 50–60% of that found in adults. This modest but statistically significant reduction could reflect a general tendency of young children to produce fewer $CCR7^{lo}$ cells in response to antigenic stimuli. This possibility is consistent with the recent finding that circulating monocytes from healthy infants and young children have a reduced capacity to produce IL-12 [96]. Moreover, in our studies of a patient with a genetic deficiency of a component of the IL-12 receptor we have previously implicated IL-12 as being necessary for the accumulation of $CCR7^{lo}$ memory CD4 T cells with Th1 function in humans [95]. An alternative and non-exclusive possibility is that the increased proportion of $CCR7^{lo}$ memory CD4 T cells in adults reflects the greater proportion of immune stimulation coming from natural infections that are effective at driving Th1 immune responses rather than from currently used inactivated vaccines that include alum adjuvant, which is a relatively ineffective Th1 inducer.

The underlying mechanism of the reduced CMV-specific Th1 response in young immunocompetent children is unknown. The finding that CMV-specific IL-2 production and CD40-ligand expression by CCR7^{lo} or CCR7hi CD4 T cells of young children is reduced, suggests that it applies to early steps by which naïve CD4^{+} T cells expand and differentiate into memory CD4 T cells. This mechanism does not appear to be attributable to skewing of the CMV-specific CD4 T-cell response towards either Th2 or regulatory cytokine production. A major question is whether this poor Th1 immune response is unique to pathogens, such as CMV, which are known to encode several gene products that could potentially inhibit the generation of CD4^{+} T-cell immune responses *in vivo* [226]. If so, this would indicate that CD4 T cells and/or cells required for T-cell activation, such as DCs, may have an age-dependent susceptibility to viral immunoevasion or viral-mediated immunosuppression that is more evident in young children. We speculate that considerable evolutionary pressure may have favored human CMV to develop and maintain the capacity to avoid inducing Th1 responses in order to spread efficiently *via* secretions during early life, as occurs predominantly in less developed countries today [201]. Regardless of the precise mechanism, this developmental limitation in adaptive immunity will need to be considered in immunotherapeutic strategies to control CMV, such as vaccination.

Immunity to live attenuated measles vaccine during the first year of life

Measles virus is a highly contagious human pathogen [227]. Although measles vaccine campaigns have been quite effective in controlling the annual epidemics in most developed countries, an estimated 800,000 children continue to die from measles annually. Measles morbidity and mortality is highest among infants who become infected during the first year of life, in developed and in developing countries [228–231]. Periodic re-introduction of measles virus is of concern in developed countries because many women are protected from measles by childhood immunization. Measles antibody titers are lower in vaccinated women than in those who had natural infection. As a consequence, the infants of vaccinated women receive lower titers of transplacentally transferred, passive antibodies against measles than infants whose mothers had natural measles infection. Earlier loss of passive antibodies at 6–9 months results in a longer period of susceptibility before the primary measles vaccine dose is given at 12–15 months of age [232–235]. Thus, understanding the obstacles to inducing measles immunity in infants vaccinated at less than 12 months old is important. To address this issue, we have compared the acquisition of the measles-specific T-cell and antibody response of infants given measles vaccine at 6, 9 and 12 months of age [232, 236–238]. Immunity elicited by mumps vaccine

was also assessed to determine whether observations about measles immunity were unique or characteristic of responses to live viral vaccines given during the first year of life.

Humoral and cell-mediated immune responses to measles and mumps vaccines given to infants at 6, 9 or 12 months of age

We found that infants in all three age groups after immunization had equivalent T-cell responses, as assessed by the cell proliferation and IFN-γ production by PBMCs to measles or mumps antigen, regardless of whether maternal antibody to the virus was present at the time of immunization [238]. A second important finding was that 6-month-old infants had relatively low measles or mumps geometric mean titers (GMTs) and seroconversion rates, but that this occurred even when no maternally-derived measles or mumps antibodies were detected at the time of vaccination. Measles-specific T-cell proliferation, as assessed by stimulation index (SI) after vaccination was > 3.0 in 72%, 69%, and 65% of 6-, 9-, and 12-month-old-infants. Mean SIs were equivalent in all three age groups. Measles-specific SIs after immunization did not differ for infants with or without measles-neutralizing antibodies when vaccinated: mean SIs were 7.3±0.9 vs 6.9±2.2 in 6 month olds and 5.6±0.8 vs 7.6±1.6 in 9 month old infants, with and without passive antibodies, respectively. Measles T-cell proliferative responses were also not affected by the amounts of measles-passive antibodies when infants were stratified by passive antibody titers >50 and <50 mIU/ml: Mean SIs were 8.2±1.9 in infants who had higher titers and 7.9±10.9 among those with lower titers. As observed after measles vaccination, mumps-specific T-cell proliferation responses were induced in infants vaccinated at 6, 9 or 12 months and were not affected by passive antibodies: mean SIs were 8.0±12.4 vs 8.7±1.6 for 6-month-old infants with and without passive antibodies, respectively, and 6.1±1.5 vs 7.1±1.1 for 9-month-old infants with and without passive antibodies, respectively (both not significantly different).

Concentrations of IFN-γ produced by PBMCs in infants given measles vaccine at 6, 9, or 12 months did not differ by age cohort. IFN-γ concentrations were 184±52 pg/ml, 349±95 pg/ml, and 115±26 pg/ml, in 6-, 9-, and 12-month-olds. IFN-γ concentrations were equivalent in infants with and without passive antibodies when immunized at 6 months of age (172±38 pg/ml vs. 273±83 pg/ml) (P=0.22), or at 9 months (188±58 pg/ml vs 326±86 pg/ml) (P=0.31). Mumps vaccination also induced IFN-γ-producing T cells in infants 6, 9 or 12 months of age. Mean IFN-γ concentrations were 239±77 pg/ml, 156±54 pg/ml, and 327±96 pg/ml, respectively. Mumps-specific IFN-γ production was not affected by passive antibodies at the time of immunization.

Although T-cell responses were not different by infant age group, measles and mumps-neutralizing antibody titers were lower after vaccination of 6-month-old infants. Measles GMTs were 120 mIU/ml (71–200

range) and 146 mIU/ml (44–490), in 6-month-olds with or without passive measles antibodies. Nine-month-old infants who had lost passive antibodies at the time of vaccination had measles GMTs of 744 mIU/ml (467–1183) compared to 180 mIU/ml (68–473) in those with passive antibodies when immunized ($P = 0.01$). Measles GMT in 9-month-olds without passive antibodies did not differ from GMT of 1210 mIU/ml (774–1893) in 12-month-olds without passive antibodies. In the absence of passive antibodies, measles GMT were lower in 6-month-olds than GMTs in 9-month-old ($P = 0.01$) or 12-month-old infants who had no passive antibodies ($P = 0.0002$). Seroconversion rates were also lower in 6-month-olds than 9-month-olds (77% vs 97% in 9-month-olds ($P = 0.02$) and in 12-month-olds (77% vs 96%) ($P = 0.01$). Only 59% of 6-month-olds had measles titers >1:120 compared to 97% and 94% of 9- and 12-month-olds vaccinated in the absence of passive antibodies (6 vs 9, $P < 0.0001$; 6 vs 12, $P = 0.0001$). Passive antibody interference alone accounted for the difference in GMT between 9- and 12-month-olds ($P = 0.003$). Six-month-olds immunized with mumps vaccine in the absence of passively acquired mumps-neutralizing antibodies also had lower GMTs than 12-month-olds; GMT were 18 (9–37), 35 (20–63), and 51 (28–89) at 6, 9, and 12 months (6 vs 12 months: $P = 0.04$).

These studies indicated that 6-month-old infants had an intrinsic deficiency in humoral immunity elicited by each of these live attenuated paramyxovirus vaccines. However, lower responses were not due solely to inhibitory effects of passive antibodies. In contrast, cellular immunity, measured as measles- or mumps-specific T-cell proliferation and IFN-γ production, was equivalent in 6-, 9- and 12-month-olds. It is important to emphasize that antiviral T-cell immunity was induced by immunization with live attenuated viral vaccines during the first year, even when passive antibodies were present. Thus, assessing the induction of adaptive antiviral immunity by antibody titers only gives an inaccurate evaluation of the immune responses of infants given live attenuated viral vaccines. Therefore, to define primary vaccine failure as a failure to develop antiviral humoral immunity, does not consider the effect of priming of antiviral T-cell responses that occurs in infants immunized during the first year of life. In addition, the commonly accepted view that passive antibodies block the infant immune response by preventing the vaccine virus replication is likely to be incorrect. The low concentrations of viral antigens in measles and mumps vaccines are not adequate to elicit antiviral T-cell responses without viral replication to enhance the antigenic signal. Other mechanisms that probably affect measles or mumps antibody production in the presence of passive antibodies include formation of antigen-antibody complexes which undergo phagocytosis, facilitating presentation of viral peptides to naïve T cells but inhibiting B-cell sensitization, and masking of B-cell epitopes by pre-existing antibodies to these regions of viral protein antigens [239].

Interleukin-12, IFN-γ and T-cell proliferative responses to measles in immunized infants

Decreased IL-12 production could impair the acquisition of adaptive immunity to measles vaccination because IL-12 promotes the rapid induction of antigen-specific T cells of the Th1 type in the naïve host. A lack of IL-12 might also favor polarization of CD4 T cells towards a Th2 type of response. Measles-specific T-cell proliferation or IFN-γ production in infants vaccinated at 6, 9 or 12 months of age were lower compared to vaccinated adults. Since limited IL-12 might explain this difference, infants and adults who had vaccine-induced measles immunity were tested using assays for IL-12 production, T-cell proliferation and IFN-γ release by PBMCs stimulated with measles [14]. Our hypothesis was that a limited Th1-like response to measles in infants could contribute to a diminished measles-specific cellular immune response. IL-12 and IFN-γ production by measles-stimulated PBMCs was measured along with measles-specific CD4 T-cell proliferative responses before and after measles vaccination of infants. These experiments showed that both IL-12 and IFN-γ release by measles-stimulated PBMCs increased significantly following immunization of infants at 6, 9 or 12 months of age. Although there were no age-related differences in IL-12 and IFN-γ production in infants of varying ages, IL-12 release by PBMCs stimulated with measles antigen was significantly lower in the infants than in adults ($P = 0.04$). In addition, PBMC cultures were supplemented with exogenous recombinant human IL-12 during the period of stimulation with measles antigen in order to determine whether the infant deficiency in IFN-γ release was caused by limited production of IL-12 by infant monocytes. The addition of rhIL-12 enhanced IFN-γ production by infant PBMC, but even when IL-12 was provided, infant T cells still made significantly less IFN-γ than adult T cells in response to measles antigen. No differences were observed in measles-specific T-cell proliferation or in the concentrations of IFN-γ produced by PBMC stimulated with measles antigen when rhIL12 was added to PBMC cultures from infants with or without passive antibodies.

In summary, diminished IFN-γ and IL-12 production in response to measles antigens may limit cellular immunity to measles in infants compared to adults. If IL-12 production is deficient, IFN-γ concentrations may not support the optimal development of infant T cells of the Th1 type reactive with measles antigens after vaccination. If responses to natural infection are similarly limited, the increased susceptibility of infants to severe measles may be attributed in part to lower IL-12 responses compared to adults, along with an intrinsic limitation in the capacity of infant T cells to produce IFN-γ [15]. These differences were detected in infants in all three age groups, including those immunized at 12 months, irrespective of passive antibody status at the time of vaccination.

Measles-specific CD4 T-cell responses in infants

Flow cytometry-based, intracellular cytokine assays have been useful for evaluating CMV immunity in infants and young children [66]. We have applied similar methods to determine measles-specific CD4 T-cell frequencies and to assess up-regulation of CD4 T-cell activation markers, CD69 and CD40 ligand and cytokine production. In studies that are now in progress, the frequencies of CD4 T cells that express CD69 or CD40-ligand after stimulation of whole blood with measles antigen *in vitro*, were not different in infants who were vaccinated at 6, 9 or 12 months. Compared to vaccinated adults, infants had fewer CD40-ligand$^+$/IFN-γ^+ CD4 T cells after stimulation of PBMC with measles antigen. Age-related differences were not detected by measuring the numbers of measles-specific activated, IFN-γ-producing CD4 T cells in 6-month-olds compared to 12-month-olds after measles vaccination, but CD40 ligand$^+$/IFN-γ^+ CD4 T-cell frequencies appear to be somewhat lower ($P = 0.05$). As expected from proliferation assays, the presence of passive antibodies to measles at the time of immunization did not affect measles-specific CD4 T-cell frequencies in vaccinated infants. These observations suggest that CD4 T cells appear to respond to measles antigen by appropriate up-regulation of the activation markers, CD69 and CD40-ligand. However, as in previous assays, infant CD4 T cells have deficiencies in IFN-γ production. Even though these responses are lower, the flow cytometry experiments indicate that immunized infants develop measles-specific CD4 T cells and should be primed to respond to an exposure to measles or to a second vaccination.

Effects of a two-dose measles vaccine regimen

An important question about the infant immune response to measles vaccination is whether age-related deficiencies of antibody production will suppress responses to re-vaccination given at later ages. To address this question, we have evaluated humoral and cellular immune responses in infants who were immunized at 6 months and re-vaccinated with measles-mumps-rubella (MMR) vaccine at 12–15 months. Among these infants, the mean SIs were 8.9 ± 3.1 after the initial monovalent measles and 11.6 ± 2.4 after MMR. The SIs in 9-month-olds were 8.2 ± 2.7 after monovalent measles vaccine and 9.3 ± 2.8 after MMR. The measles-specific T-cell proliferation responses measured after MMR was given to infants who had received monovalent measles vaccine at 6 or 9 months of age were higher than those of 12-month-olds who had received MMR only (6 vs 12 months, $P = 0.008$; 9 vs 12 months, $P = 0.04$). In addition, the measles-neutralizing antibody responses increased after MMR to GMTs of 1487 mIU/ml (884–2497) in the 6-month-olds and 1994 mIU/ml (809–4920) in the 9-months-olds. All infants developed titers ≥ 120 mIU/ml after MMR. These

studies showed that an early two-dose measles immunization regimen elicited measles-specific T-cell responses in infants as young as 6 months, which were associated with enhanced humoral responses after receiving MMR at 12 months of age. Further, seroconversion rates and measles GMTs after second doses of measles vaccine were not affected by initial interference from passive antibodies with the responses of 6- or 9-month-old infants to the first measles vaccine dose.

Summary

These studies of neutralizing antibody responses to measles and mumps vaccination of 6-month-old infants imply an intrinsic deficiency in humoral responses in this age group which are separate from the well-known effects of passive antibodies on initial seroconversion and GMTs. Importantly, measles- or mumps-specific T-cell proliferation or IFN-γ production showed no age-related differences when vaccines were given at 6, 9 or 12 months. Furthermore, passive antibodies had no effect on T-cell proliferation or cytokine production by PBMCs stimulated with measles or mumps antigens. Nevertheless, comparisons of responses in vaccinated infants and adults revealed significant differences, including lower measles-specific CD4 T-cell proliferation and IFN-γ and IL-12 production. Infants had also lower frequencies of $CD69^+$/IFN-γ^+ CD4 T cells and fewer $CD40L^+$/IFN-γ^+ CD4 T cells than adults after measles stimulation *in vitro*. Mechanisms that may account for less robust humoral immune responses in younger infants may include limited IFN-γ production by helper T cells, decreased measles-induced IL-12 production by APCs affecting induction of CD4 T cells and limited antigen-induced up-regulation of CD40-ligand which could restrict interactions of helper T cells and B cells. These analyses of infant responses to live attenuated viral vaccines suggest that concepts about antiviral immunity in the first year of life should be re-examined, e.g., passive antibody interference should be re-defined since antiviral CD4 T cells are elicited in the presence of passive antibodies with virus-neutralizing function. Neutralization of live viral vaccines by maternal antibodies appears to be an oversimplification of the biological events. Instead, specific mechanisms that modulate antibody production need to be identified, independently of the induction of antiviral T cells. Re-thinking the biology also requires considering an alternative paradigm in which inhibition of antibody production by passive antibodies is not "interference", but modulation to avoid antibody excess and immune-mediated disease in early infancy. As noted, priming of antigen-specific helper T cells occurred in some infants who did not develop measles or mumps antibodies, indicating a need to re-define primary vaccine failure since virus-specific T cells provide these infants with some measure of active, antiviral adaptive immunity. Induction of partial immunity may explain the clinical experiences in which early immunization

has been associated with protection from severe measles disease and mortality despite the occurrence of breakthrough infections [240]. Although measles-specific responder CD4 T-cell frequencies, T-cell proliferation, and IFN-γ and IL-12 production were lower in infants than adults, a second dose of measles vaccine at 12–15 months increased antiviral T-cell responses to measles in infants who were vaccinated at 6 or 9 months, and resulted in equivalent seroconversion rates. Our hypothesis is that since T-cell immunity is induced under the "cover" of passive antibodies, repeated doses of live viral vaccines given during the first year of life constitute a "prime" and "boost" strategy that mimics the pattern of exposure and re-exposure to pathogens during infancy and early childhood, which was a common occurrence before live viral vaccines were introduced to protect against measles, mumps and other childhood infections.

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Regulatory issues

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Regulatory issues in the development of new vaccines with a special emphasis on safety aspects

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Introduction

Vaccines represent a major weapon against many infectious diseases. More recently, vaccines have demonstrated promising methods in the prevention or treatment of other types of disorders such as cancer and autoimmune diseases. Their composition has significantly evolved from the whole microorganism approach (attenuated or inactivated) to the use of crude fractions or purified parts of these organisms (polysaccharides and toxoids) through to protein-polysaccharide conjugates and now to recombinant proteins or polypeptides. Gene transfer products (naked DNA and vector-based vaccines), which are currently in pre-licensing phases, also represent a new approach in vaccinology [1]; they are already successfully used as veterinary vaccines for rabies or distemper. Therefore vaccines are multifaceted products and also act through a highly complex mechanism in which the antigen(s) by itself is not the final acting component of the immune response. Vaccines produce antibodies or activate T cells that are the actual effectors. Obviously, this complexity raises regulatory issues associated with the efficacy and safety assessment of vaccines.

Vaccines fall within the scope of medicinal products (as defined by the amended Council Directive 65/65/EEC [2]) even if they are considered as a separate category as illustrated by the dedicated Office of Vaccines Research and Review within the U.S. Food and Drug Administration – Center for Biologics Evaluation and Research (FDA-CBER) organisation. Therefore they must undergo strict non-clinical and clinical safety evaluation before licensing in line with what is performed with drugs. This classification as a medicinal product associated with the specific status of vaccines implies that documents for pharmaceuticals in general and specific vaccine guidelines provide regulatory guidance for vaccine development. For instance, the WHO publishes appropriate vaccine guidelines covering the nonclinical and clinical development of vaccines only [3, 4].

One of the major differences between vaccines and other pharmaceuticals is the fact that they are given to healthy people, and predominantly to

children. As a consequence, safety is a major focus in the assessment of vaccines.

The risk-benefit ratio of vaccination in general is very different between developing countries and developed countries. The recent Rotashield vaccine is a perfect example of this statement. The product has been withdrawn in the US after cases of intussusception in temporal relation to the administration of the vaccine. The withdrawal was a worldwide withdrawal although the benefits of the vaccine in developing countries are still obvious.

The lay public is expecting to receive vaccines which bear a zero risk while the authorities as well as the manufacturers have to deal with an increased number of liabilities. As a consequence, the level of safety requirements has increased tremendously. The time to licensure and the price of the vaccines have increased in parallel.

Vaccine life can be split in three stages: development, licensure and post-licensure. In this document, regulatory aspects will be considered for the development stage with a special emphasis on the nonclinical safety issues as the clinical development will be reviewed in some detail in other sections of this book.

Characterisation of starting materials, production process and final product

Several guidelines present the requirements for the characterisation and control of biological products and/or vaccines prior to licensure. The majority are edited under the International Congress on Harmonization (ICH) process [5–9], by the WHO [4] or by Pharmacopoeia (e.g., European Pharmacopoeia).

Vaccine characterisation starts before the first clinical trial and continues during the clinical development. Based on the test used to characterise the vaccine and also considering previous experience from vaccines from the same category, a range of quality control tests is selected. Vaccine specifications are established from this relevant set of tests. In line with the good manufacturing practice, all these tests must be standardised and validated. Validation may be only partially achievable at the beginning of the vaccine development when innovative techniques are used and the vaccine candidate is not yet in its final formulation.

The particularities of each vaccine imply a product-based strategy for quality control and characterisation and regulatory authorities are able to accept this product-design approach. However, a seed-lot system (for culture of micro-organisms) or a cell-bank system (for culture in cells) is applied as recommended by the European Pharmacopoeia [10] to ensure uniformity and full traceability. The system starts with the Master seed lot (or Master cell bank), continues with the Working seed lot (or Working cell bank) and ends with the final bulk which is filled to obtained the final lot

or batch of vaccine. However, when primary cells or embryonated eggs are used, characterisation has to be repeated for every new production run [11].

Quality control includes first the evaluation of the raw or starting materials. This evaluation can start with a supplier audit. Any unwanted endogenous or extraneous agent should be evaluated [12, 13]. For instance, the risk of prion contamination seriously affected the biotechnology industry. The bovine spongiform encephalopathy (BSE) and the related new variant of Creutzfeldt-Jacob Disease (CJD) led to strict rules concerning materials of animal origin. Not only should vaccine manufacturers use BSE-free countries (New Zealand, Australia) whenever feasible for their materials of animal origin, but they should also document the full process from the origin of bacterial or viral seed to their current products. Any undefined step with potential contamination by products of animal origin can require re-derivation of the seed using methods such as RNA transfection.

The absence of other adventitious agents, and particularly viruses, is an essential safety requirement for all biotechnological products. One of the technical difficulties of these tests for live viruses, particularly in early development phases, consists in the requirement for a pre-neutralization of the vaccine virus using specific antibodies. Details about essential phases of viral safety can be found in quality guidelines [8, 12, 13]. Animals are still used for these tests but new *in vitro* methods have been developed recently in addition to animal tests. For instance, the polymerase chain reaction (PCR) amplification can detect fragments of viral contaminants and the product-enhanced reverse transcriptase (PERT) assay is used to identify traces of retroviruses [11].

In addition to viral safety tests, any new cell bank should be studied for their tumorigenicity in chemically or genetically immunosuppressed rodent models (i.e., athymic mice, newborn rodents treated with antithymocyte serum or globulin or thymectomised and irradiated mice that have been reconstituted with bone marrow from healthy mice) with a comparison to a reference tumorigenic cell line. The observation of tumors at the injection site and potential metastases are the endpoints of these tests. *In vitro* methods are also proposed [12]. As a consequence, a limit is required for residual substrate-cell DNA from a tumorigenic cell line (e.g., 10 ng per single human dose based on the European Pharmacopoeia). Bacterial and fungal contaminants are also investigated. Mycoplasmas are carefully screened by culture or indicator cell culture methods [14].

The second quality control phase corresponds to the in-process control. Appropriate in-process controls may eliminate the need for tests on the final product and can allow a reduction of animal use for batch release. A validation of the manufacturing process will concern all aspects of the vaccine manufacturing process including equipment, facilities, and personnel; it allows production of consistent lots. The completion of this validation generally requires at least three released batches.

The quality of the process is also supported by compliance with Good Manufacturing Practice (GMP) and clinical batches should be issued by GMP facilities. Julie Milstien [15] perfectly illustrated the parallel between the "Cutter incident" which occurred in 1955 and then the implementation of GMP rules including those for the development of clinical batches of vaccine. Unfortunately, 260 cases of paralytic polio and 11 deaths were caused by an inactivated polio vaccine in the 50s. Not only did this case result in stricter controls and regulation but also validation, facility inspection, quality assurance implication in protocols and reports required by the GMP safeguarded against such incidents.

Finally quality controls are performed on the final bulk and/or final batch; these include sterility and physico-chemical analysis. Several vaccines still use attenuated or inactivated micro-organisms. Specific toxicity tests are performed as part of the range of quality control tests to verify the attenuation or the inactivation of the micro-organism. As for other tests, *in vivo* methods were the reference technique and are being progressively replaced by *in vitro* techniques [16]. For instance, the rabies vaccine inactivation was tested by the intracerebral route in mice and can be tested on cell culture for non-adjuvanted formulations nowadays. Oral polio vaccine is still tested by an intraspinal route in monkeys for neurovirulence, although alternative methods (molecular-based assay (MAPREC) and transgenic mice expressing the human polio virus receptor) are currently being evaluated and used [16–18]. Neurovirulence tests are also considered for non-neurotropic vaccines but not as a batch-release test. Specific toxicity tests are also used to control the inactivation of bacterial toxins (e.g., diphtheria and tetanus toxins tested by the intraperitoneal route in the guinea-pig or by *in vitro* methods using Vero cells and *Bordetella pertussis* toxin tested by the same route in mice). As a complement to this specific toxicity, abnormal toxicity (also called general safety) tests are also performed at various steps of the vaccine production process (final product, and, in some cases, bulk and primary seed) by intraperitoneal injection of the product in mice and guinea-pigs. Mortality and body-weight decrease are recorded during these tests. These abnormal toxicity tests in animals are intended to detect any toxic substances accidentally introduced in the final vaccine formulation. However, they can be challenged from an ethical perspective and could be irrelevant if the vaccine components are susceptible to triggering false positive reactions due to their reactogenicity by this intraperitoneal route of administration. Therefore, abnormal toxicity tests are removed from several vaccine monographs [19].

To complete this section on safety evaluation, as part of the quality assessment of vaccines, pyrogenicity tests should be carried out particularly for polysaccharide vaccines. The use of the rabbit pyrogenicity test decreases in favor of the *Limulus amoebocyte* lysate test (LAL).

In addition, stability studies are performed not only on the final batch but also on process intermediates using several of the tests mentioned

above and, more particularly, safeguarding of potency should be verified.

It would be inappropriate to define a universal list of quality tests due to the diversity of vaccine approaches, each of them being associated with a specific development strategy. Therefore the tests selected for the specifications of a viral vector-based vaccine are presented in Table 1 as an example of the application of the above-mentioned principles. Tests are done on the cells used to produce the virus (i.e., avian cells) and on different steps of the viral suspension preparation from the bulk viral suspension to the finished product.

Results from these release tests (i.e., compliance with specifications), combined with the release of the raw materials and a careful review of the production process and associated data (e.g., data from the environment) allow the release of a vaccine batch for clinical trials. By performing these tests, the manufacturer should verify vaccine safety aspects such as product sterility, but also demonstrate that the manufacturing process is consistent.

Non-clinical safety evaluation

The most exhaustive description of the non-clinical safety requirement is probably included in the recent WHO guideline [4]. This document is the result of concerted work by the WHO, drug agency and industry representatives and represents a global consensus of the diverse regulatory bodies. The description of the main non-clinical safety studies included in this section will comply with this document. However, guidelines applicable to all pharmaceuticals including biotechnology-derived products provide helpful support, such as the ICH S6 guideline entitled "Preclinical safety evaluation of biotechnology-derived pharmaceuticals" [20]. It clearly explains the flexible strategy recommended for biotechnology-derived pharmaceuticals, including vaccines. The scope of this document includes recombinant protein vaccines but does not cover conventional bacterial and viral vaccines or DNA vaccines.

With regard to the specific guidelines, the European document by the European Agency for the Evaluation of Medicinal Product (EMEA), Committee for Proprietary Medicinal Product (CPMP), December 1997, entitled "Note for guidance on preclinical pharmacological and toxicological testing of vaccines" [21] is the only finalised guide solely dedicated to the pre-clinical safety evaluation of vaccines published by the European agency. There is no such guideline in the US and the "Guidance for industry for the evaluation of combination vaccines for preventable diseases: production, testing and clinical studies" [22] only includes a short section dealing with non-clinical studies.

Interestingly, the FDA has recently released a draft guidance document on reproductive toxicology for vaccines intended for women of child-bear-

Table 1. List of the quality control tests selected according to the different manufacturing steps for the specifications of a viral vector-based vaccine produced on avian cells.

Control cells		
• Observation	• Control cells: Supernatants Bacterial and fungal sterility test	• Test for extraneous agents using cells: - Chick embryo fibroblast cells - Continuous simian kidney cells - Human diploid cells
• Test for haemadsorbing viruses	• Test for mycoplasmas using microbiological culture method • Test for mycoplasmas using indicator cell culture method	• Test for avian leucosis viruses • CoRT test (research of exogenous avian retroviruses on CEF cells)
Viral suspension		
• Detection of reverse transcriptase by semi-nested PCR (PERT assay)	• Transmissibility assay on human cells	• Identification
• Bacterial and fungal sterility test	• Test for extraneous agents using animals: - Mice - Suckling-mice - Guinea-pigs - Rabbits	• Test for extraneous agents using cells: - Continuous simian kidney cells - Human diploid cells - Chicken embryo cells
• Test for mycoplasmas using microbiological culture method	• Test for extraneous agents using embryonated eggs: - Allantoïc cavity - Yolk sac	
• Test for mycoplasmas using indicator cell culture method		
Clarified harvest		
• Bacterial and fungal sterility	• Virus concentration	
Final bulk product		
• Bacterial and fungal sterility	• Assay of residual antibiotic (neomycin)	• Test for removal of intact cells
Filled product, finished product		
• Appearance • Dissolution time • Appearance after dissolution • Residual moisture content	• PH measurement • Osmolality measurement • Bovine serum albumin content • Bacterial and fungal sterility	• Endotoxin content • Abnormal toxicity test • Identification • Virus concentration

ing age and those which may be used to immunise pregnant women [23]. The recommendations have been adapted from document ICH S5a in order to include the specific properties of vaccines.

For vaccines considered to be in the gene therapy or gene transfer medicinal product category (e.g., pox vector and naked DNA), the following specific guidelines are of use:

- FDA, CBER December 1996: "Points to consider on plasmid DNA vaccines for preventive infectious disease indications" [24]
- FDA, CBER March 1998: "Guidance for human somatic cell therapy and gene therapy" [25]
- EMEA, CPMP April 2001: "Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products" [26].

In addition, the EMEA has recently released a document for consultation called "Guidelines on adjuvant in vaccines" [27].

Guidelines and position papers are regularly published or amended by drug agencies, particularly for new therapeutic approaches or very specific situations such as the development of a smallpox vaccine within the framework of government efforts to combat bio-terrorism or the development of pandemic flu vaccines [28, 29]. The list of documents given is subject to modification and it is recommended that the latest version be checked on the drug agency's web site.

Outline of the proposed studies

With regard to the toxicological studies, animal models remain the best option for mimicking the human situation. Despite many efforts to develop *in silico* and *in vitro* models, such replacement models remain of limited use as they are not able to reproduce the complexity of the human immune system.

Thus, a critical challenge is the identification of the "relevant" animal model. The criteria used include the following (in order of importance): development of an immune response similar to the expected human response after vaccination, demonstration of a similar binding profile for the induced antibodies [30, 31], susceptibility to the targeted pathogen and, perhaps the most difficult to fulfil, the ability to develop exacerbation reactions [32].

These criteria imply the need to be able to generate and interpret immunogenicity data in the species used for toxicological studies. Rats and monkeys remain the most frequently used species. Recently, the use of newborn or aged rodents has been considered for the evaluation of vaccines intended for neonates or elderly people, as the immune responses of these populations present some peculiarities [33, 34].

The evaluation of the potential intrinsic (direct) toxicity of the test article is generally the main outcome of single-dose toxicity studies; therefore the relevance of species selection is less critical than for the subsequent animal studies and rodents are used. The need for this type of study can be challenged. The remarks written about general safety tests and the efforts made to reduce this type of animal study are also applicable to this type of

study. Repeated-dose toxicology studies are regularly the pivotal non-clinical safety study before the first human administration.

However, if the single-dose study is the pivotal study supporting a clinical trial, the design should be modified to include more parameters (i.e., clinical pathology and microscopic examination of a limited list of tissues).

The repeated-dose study or studies are considered to be pivotal and should be performed in full compliance with Good Laboratory Practice. Several criteria have to be met in the design of this type of study. The fundamental selection of the animal model was mentioned above. The route of administration and the dosing regimen should mimic the proposed human schedule, although the time between injections can be reduced for practical reasons (e.g., 3 weeks). This is probably the easiest way to fulfil the "be relevant" principle. Dose levels are also widely discussed. Indeed, vaccines and their potential adverse effects do not always follow a linear relationship. The most common approach is to test one full human dose per animal when it is technically feasible. When two dose levels are tested, the lower should correspond to the "pharmacological" dose (the dose known to induce a significant immune response in the selected species) and the higher should be equal to one human dose. Parameters monitored include clinical signs, ophthalmology, clinical pathology (haematology and serum clinical chemistry) and at necropsy, organ weights, gross and histopathological examination. In the interests of reducing animal use and study costs, the assessment of local tolerance during either a single- or a repeated-dose toxicity study can be made when the route of administration used is the same as for humans. In order to take the prolonged action of vaccines through the immune response into account, animals can be split into two sub-groups, the first sub-group sacrificed immediately after the last administration and the second fifteen days later as close as possible to the immune response peak.

During the in-life phase of repeated-dose studies, additional parameters of safety pharmacology, such as body temperature, electrocardiogram and blood pressure, could also be considered. Their inclusion is recommended for cost and ethical reasons as long as they do not interfere with the other parameters. The traditional range of safety pharmacology tests does not seem relevant for vaccines. It was requested as an exception in order to investigate mechanisms potentially involved in human adverse reactions (e.g., convulsion animal models). Efforts to develop vaccine-relevant safety pharmacology parameters, such as interference with existing immune disease, are encouraged.

Indeed, in all pivotal studies, a basic screening of the immune system should be performed (e.g., histopathology of the lymphoid tissues and blood cell counts). Additional immunological indicators (e.g., cytokine production) can be requested on a case-by-case basis, but interpretation of the results remains difficult.

Vaccination of women of child-bearing age (e.g., rabies and meningitis vaccines) and even pregnant women (e.g., influenza vaccine) is common [35,36] and requires an assessment of the embryo/foetal and perinatal toxicity before licensing. Not only is successful gestation dependant on the status of the immune system [37] but antibodies from pregnant animals can also cross the placenta [38] and could have supposedly harmful effects on the foetus.

The classical division of reproductive toxicology studies into 3 segments as used for new chemical entities is not wholly applicable. Treatment design including immunisation(s) before mating is recommended for vaccine embryo-foetal toxicity studies in order to provide antibody exposure during the entire embryo-foetal period. Post-parturition immunisation is suggested for a sub-group of females not submitted to caesarean delivery for foetus examination whose pups will be necropsied at the end of the lactation period in order to study the effects of antibodies in the newborn. A preliminary study to define and justify the laboratory animal species chosen should demonstrate appropriate antibody transfer with exposure of the embryo to vaccine-induced IgG. This is recommended because immunoglobulin transfer during gestation can render the animal model more susceptible to developmental effects following pre-natal exposure to the vaccine immunoglobulin. As part of the study in the main species, foetal examination, developmental monitoring of the litters and pup necropsy should be included.

The above-mentioned studies constitute the core package of the non-clinical safety investigations and even if their design remains flexible, their completion or the accessibility to equivalent data are generally mandatory.

In addition, more product-specific non-clinical safety studies can be judged necessary according to the vaccine category or published safety concern. For instance, hypersensitivity and auto-immune reactions are the most commonly perceived adverse effects associated with vaccination [39, 40]. The ability of animal models to predict these adverse reactions is a real challenge as they are likely to be rare. Systemic anaphylactic models and biological products, including vaccines, have always been a subject of controversy. However, validation data were obtained mainly from chemicals and not from pharmaceuticals. One can regret the lack of research work in this field which would put us in a better position to decide whether to study hypersensibility reactions on new vaccine components.

To focus on auto-immunity, there are two aspects to be considered: the induction *de novo* of auto-immune disease, and the exacerbation of existing auto-immune disorders [41]. In the first category, auto-immune reactions induced by molecular mimicry occur when the host and the microbial/vaccine determinants are largely similar and present in such a way that there is a break in immune tolerance. This molecular mimicry was also suspected with some new vaccines, such as the recently commercialised Lyme vaccine, where the selected antigen, namely the outer-surface protein Osp A from

Borrelia burgdorferi, presents sequence homology with human LFA-1, and it is suspected that it triggers treatment-resistant Lyme arthritis [42]. Risk of molecular mimicry was also considered for a polysaccharidic vaccine against meningitis. The Group B meningococcal capsular polysaccharide used for this vaccine is a polymer of sialic acid which mimics the polysialylated form of neural cell adhesion molecules present particularly on mammalian embryo cells [30]. Recent advances in computer software (e.g., LifeSeq from Incyte) and the availability of the human genome sequence allow rapid comparison between the protein sequence alignment of a vaccine antigen and a host protein. However, results using these tools present some limitations as predictive methods. For instance, for B-cell epitopes, sequence comparison will look for primary structure mimicry but similar antigenic surfaces (conformational mimicry) is technically very difficult or even impossible to detect. However, T-cell epitope mimicry requires not only an appropriate presentation of the identified common peptide by antigen-presenting cells but also the presence of auto-reactive T cells, which can recognise the mimicking peptide-HLA complexes.

From these hypotheses, a recommended strategy would be to avoid any vaccine antigen presenting a mimicry with a host antigen involved in an auto-immune disease. In any case, surrogate markers can be included in the protocol of toxicology and clinical studies to detect any sign of pathogenic auto-immune response.

The second identified category of auto-immune adverse effects is the exacerbation of a pre-existing pathological condition as reported for influenza infection and multiple sclerosis [43]. Animal models for auto-immune diseases (e.g., MRL/Mpj-lpr, NZB/NZW, SJL, NOD mice and BB rats) have been proposed [44] but we do not yet have sufficient validated data. Moreover, observations of biological markers for auto-immune or hypersensitivity reactions are not necessarily linked to pathogenic consequences. For instance, and this is an important point, the presence of auto-antibodies does not necessarily indicate the induction of auto-immune disease.

In order to cover all safety issues related to vaccines, the evaluation of new adjuvants or any other components of the vaccine formulation should also be considered and dedicated studies are usually required. Novel adjuvants are classified as active compounds and they are evaluated as new chemical or biological entities independently from the vaccine formulation. Recommended studies are listed in the recent EMEA guideline [27]. These studies include genotoxicity assays and repeated- and single-dose studies. Specific attention is given to pharmacokinetic studies as illustrated by the work done for *E. coli* and cholera toxins administered by the intranasal route [45]. Specific studies are added, taking into consideration the mechanism of action of the selected adjuvant. For instance, an adjuvant derived from LPS should have been modified to avoid LPS toxic activity. Pre-clinical tests such as endotoxin shock in the mouse should confirm the inactivation of intrinsic properties.

The hard task of removing mercury-containing preservatives because of their potential neurotoxicity emphasises the need to guarantee the safety of extraneous components added to a vaccine formulation. Experimental data should be collected if the scientific literature does not cover all safety aspects.

Conclusion

The development of vaccines has reached a high level of complexity where the safety concerns need to be addressed correctly. Regulatory guidelines and recommendations provide a framework to the manufacturer to develop his products. The final goal is the release and license of high-quality vaccines.

However, for several reasons (desire of zero risk from the lay public, liability issues for the authorities and manufacturers), the weight given to the collected data is not always put into the right perspective concerning the risk-benefit ratio.

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Clinical trials

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Clinical trials in developing countries: ethical issues

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Introduction

The ethical issues one needs to consider when doing clinical trials in developing countries are so manifold that one could write an entire book on them. In this chapter, we concentrate on the main issues which we have had to consider beforehand (capacities of ethical review committees, truly involving community in research, information process) and during a clinical vaccine trial when things do not go according to plans, when ethical issues arise which need immediate solving. The main ethical principles and guidelines developed are accessible in the already published literature and websites listed in the references. How to apply these guidelines in practice is the golden skill one only learns in the field, by doing, by putting in practice those principles in dialogue with the different stakeholders, and especially the population where trials are being conducted.

Trial design and ethics

Where resources are limited, the decision to do a trial has to be carefully thought through. The choice of trial design, sample size and level of power has ethical implications. It is said that bad ethics makes bad science, and *vice-versa*. Therefore, the trial design has to be fitted to the primary question posed by the research. The primary objective of the trial and the limits of confidence acceptable to its statistical significance dictate the sample size needed for the trial. It is unethical to purposely underpower a study by too small a sample size. Also, an underpowered study implies that an opportunity is lost to do another, possibly better designed, scientifically more robust trial in the community. The gold standard of new vaccine research is the individually-randomized, controlled, double-blinded study. Ideally, the control antigen is a placebo so that absolute rather than relative immunogenicity and efficacy comparisons can be made. The use of a true saline placebo

in poverty-stricken conditions has been strongly criticized; the argument has been that at least some protection should be given to the control group as well. Yet if it is very likely that the expected price of the new product will prohibit the introduction of the intervention in these conditions unless the new product is highly efficacious, it is counterargued that it is unethical not to find out the absolute efficacy, thus aiding local decision-makers with relevant data arising from their own circumstances. An often-used compromise is to provide some form of additional benefit to the individuals participating in the trial after the trial is over (see discussion in point "Ethical issues relative to informed consent"). Another way of looking at the benefits given to the trial participant is to consider the care given during the trial, i.e., other additional vaccines, diagnostic tests and medical care during outpatient or hospital visits that the participant is entitled to during the trial. An example is provided by a pediatric pneumococcal conjugate vaccine trial in Asia, the investigators of which were advised by the reviewing supranational ethical committee to provide a comparison between the currently licensed 7-valent vaccine, rather than a saline placebo, and the new 11-valent one. The rationale given was that it would have been unethical not to provide a treatment, i.e., 7-valent vaccine, which already was licensed in some western countries. The investigators argued that even if the 7-valent vaccine was available in some countries, it was not available in the country where the study was to be conducted, and even if it was, it would not be available to the vast majority of children. Also, the researchers argued that given their provision of all other EPI vaccines as well as diagnostic and medical treatment when a trial subject sought medical care for the trial clinical endpoint conditions (i.e., acute respiratory infection, pneumonia, meningitis, sepsis), they provided considerable benefits to all participants. Thus, in the absence of any other non-confounding alternative study vaccines, the saline placebo was accepted as the control vaccine to the study which held X-ray-positive pneumonia as its main endpoint.

The definition of an adequate and/or a standard level of care given to trial participants during the study has been the centerpiece of argumentation in several HIV trials carried out in developing countries (i.e., is the measure for "standard" taken from rich or poverty-stricken settings?). While trials have the general tendency of improving health care in the area where they are conducted, it is also usually not acceptable that trials fundamentally change the care-giving practices for those participating in the trial and thus further create obvious inequality in the trial area. On the other hand, it is clear that any adverse health problem caused by the trial product has to be properly taken care of and compensated for during the course of the trial. It is the role of the Independent Data Safety Monitoring Board (DSMB), among their other duties, to safeguard the trial participants in this respect and provide neutral technical advice to the investigators, even if the ultimate responsibility for the trial participants lies with the investigators and the sponsors.

Capability-building for ethics review committees

In the past decade, there has been an increasing trend of outsourcing clinical trials to developing countries. Admittedly, there are several good reasons for such decisions. For one, sick patients, who are usually the targets of research, are so many in the developing countries, that it significantly shortens trial recruitment and enrolment time. Other trial-related costs, including incentives, treatment and hospitalization, as well as payments for research investigators, are considerably lower in developing countries. Increasingly, however, this trend has given rise to what is perceived as the moral controversy of conducting clinical trials in countries where ethical review committees (ERCs) may not make an effective evaluation of research proposals or at worst are not available at all [1–4].

Requirements of an effective ethical review

We consider the following to be necessary for effective ethical review of research proposals:

1. need for trained and competent ERCs;
2. presence of national regulations and ERC-specific operational guidelines;
3. community representation in ERCs; and
4. independence of ERCs.

Trained and competent ERCs

The number of studies conducted in developing countries has multiplied considerably over the last decade. No parallel development and organization of ERCs, however, has taken place in these countries. There are still some countries which do not have ERCs either at the national or institutional levels, causing reviews to be done either by appointed officials of the health ministry or by *ad hoc* committees. In a survey of health researchers in developing countries, nearly half of the respondents reported that their studies did not undergo any form of review – technical, scientific or ethical – by the health ministry of the country [5]. Very recently, an uproar was raised in India over the conduct of a clinical trial which evaluated the ability of a drug to induce ovulation, indicated for the treatment of breast cancer in post-menopausal women, without ethical approval [6]. And yet, India just recently came up with national ERC guidelines.

In countries with functional ERCs, the concern is about the quality and effectiveness of the ethical reviews made. Observations document that developing-country ethical reviews are done perfunctorily, for formality or rubber-stamping purposes, with minimal or practically no ethical questions

raised. A review of the status of health ethics of Asian countries revealed that the existence of national ethics bodies did not equate at the institutional levels with effective evaluation capability [2]. More provocative is the commentary that developed-country ERCs have a "low regard" for the quality or "cosmetic" reviews made by developing-country committees [1]. These observations, whether accurate or not, connote that there may be a number of "unethical" studies being carried out in the third-world countries, because of deficiencies in ethical reviews. This anxiety is further fueled by the increasing complexity and sophistication of new research topics, in the areas of genetic epidemiology and molecular biology for instance, which these ERCs may be too technologically naïve and inexperienced to review effectively.

The different international guidelines for human research are very specific about the need for ethical review as an overt translation of the application of ethical principles. These documents include proposals for the composition, roles and responsibilities of ERCs. There is an urgent need for developing countries to heed this requirement as an initial step towards guiding and regulating the research they will host and participate in and to preclude any possible exploitation of their populations due to inequities in socio-economic conditions and access to health services.

The establishment of ERCs is not sufficient by itself. ERCs should be dynamic, i.e., conversant with the guidelines and their provisions. Its members should be trained in review processes and the mechanisms they can employ to guarantee that only ethically acceptable research is done [2, 7]. ERCs must have a clear appreciation of the relationship between scientifically sound and ethically acceptable research. Like any other ERCs, developing-country ERCs also need training in basic research ethics and should take part in continuing education to keep abreast with ongoing ethics and scientific discourses. There are several online ethical review courses hosted for regulatory authorities, medical schools and the like. Often these are, however, designed to satisfy institutional requirements and rapid accreditation of investigators rather than to encourage critical appraisal of the elements of research proposals. Training programs wherein guidelines are interpreted and applied in case analysis and discussions are needed. These exercises provide insights into what the actual ethical review process is about, how issues should be addressed and conflict resolutions should be made and the difficulties the application and interpretation of the ethics guidelines create in real-life situations.

Presence of national regulations and ERC-specific operational guidelines

The international guidelines for the ethical conduct of research are intended to ensure the protection of the research participants from exploitation

and harm. Some of the provisions of the recently revised Declaration of Helsinki (DoH) [8] are still heatedly debated because of varying interpretations, their impact on future research, particularly those with public health implications, and because they do not take into account the unique conditions of developing countries [2, 9]. Also, research is increasingly becoming more complicated and complex. This scenario requires that countries take explicit and unambiguous positions in relation to some provisions in the international guidelines, such as the use of placebos in clinical trials, or whether the "best currently available treatment" is synonymous with the "locally available treatment." This situation may also spell the need for less-developed countries to create a separate set of guidelines as aptly demonstrated by India's development of its own Ethical Guidelines for Social Science Research in Health.

Despite the fact that international guidelines are based on commonly agreed ethical principles, these are open to varying interpretations by the bodies and committees who apply them. This is brought about by varying cultural and value orientations, educational background and access to information. Thus, developing-country ERCs may interpret and apply these intuitively in the review and evaluation of research proposals, as ERCs are very recent and an evolving phenomenon in the developing countries.

One way around this is apparently the establishment of national guidance which will incorporate and reflect a country's local norms and value systems, and procedurally involve wider community participation and consultations. If at all feasible, the practical and operational aspects of its application, such as the selection of ERC chair and members, how administrative and financial support may be provided to ERCs, or how issues may be resolved, should be elucidated. In addition, ERCs at both the national and institutional levels should be urged to develop their own manual of operations or procedures. This could further enhance their appreciation and application of both the international and national guidelines. Its availability provides not only a definite and explicit framework, but also, and more importantly, a set of standards within which ERCs could function and operate. These approaches may pave the way towards a more effective, efficient, as well as a more homogeneous and harmonized ethical review, which is transparent to all parties involved. These reduce the possibility of divergent opinions and different opinions between ERCs, particularly with the escalating multi-center studies, and thus multi-ERC reviews.

Community representation in the ERCs

The potential composition of ERCs is indicated in several documents but most prominently in the guidelines developed by the World Health Organization (WHO) [10]. The purpose of a multidisciplinary and wide diversity in the composition of the committee is that the competency and

experience that each member brings into the protocol reviews somehow is a guarantee that all possible ethical and moral issues arising from the scientific and logistics plans described in the research protocols are identified, addressed and appropriately decided on. Ideally the review process should take place in two steps: the scientific soundness should be reviewed by an independent, scientifically qualified review committee, and only if the science is found sound, should the multidisciplinary ethics review follow.

In practice, however, ERCs may be composed primarily of institutionally-affiliated scientists with limited or restricted community representation. In many cases as well, the so-called community representative does not truly exemplify the modal community members, e.g., journalists, and thus may not truly represent their best interests and well-being [11].

More reflection on the selection of community representatives to the ERCs should occur. If a permanent representative is difficult to obtain, then provisions should be made that *ad hoc* representatives of the community where the research will be conducted may be invited during the protocol deliberations. For instance, gay activists or gay HIV-affected individuals could serve as community representatives for an HIV clinical trial intending to recruit participants from the gay community. In this manner, the ERCs are assured that representatives will have a primordial interest in truly protecting the community to which they belong. For instance, they should be able to state whether proposed inducements or benefits are appropriate or whether safeguards for data and personal information are adequate. Community representatives will naturally need orientation and training to be able to participate meaningfully in ethical reviews.

Independence of ERCs

Among the challenges confronting developing-country ERCs is their ability to operate independently of the institutions to which they are affiliated. Independence here is defined as the ability of ERCs to conduct intelligent and effective reviews free of administrative and financial pressures and free from conflicts of interests among its members. This is addressed by the lay membership in the committee, by establishing transparent provisions of lines of accountability and conflicts of interests and providing ERCs with their own logistical support.

The Nuffield report [3] documents that the estimated direct and indirect cost for operating ethics committees in the United Kingdom may amount to £36,000 per annum, while that in the US would reach as much as US$ 500,000 per annum. These are staggering amounts which ERCs in developing countries can ill afford. NBAC [4] learned from its commissioned studies that ERCs in developing countries did not have budgets or access to resources to support the functions of the committee, such as salaries of members, meetings, secretarial assistance, training and networking with

other ERCs, and the like. In effect, the institution itself absorbs the operating costs of its ERCs, which although small, may be considerable in relation to their limited resources.

Convincing scholars, lawyers and members of the religious groups or community to sit in the ERCs on a voluntary basis or with minimal incentive, is quite difficult to do. Thus, several developing-country ERCs include researchers and scientists as its members. They perform ethical reviews on top of their research and teaching duties, without any additional compensation for their time and effort. Evidently, questions of conflicts of interest may be raised when these same ERC members present their own proposals or are involved in whatever capacity in the research proposals being evaluated. However, this issue can be addressed by establishing transparent provisions on how the ERCs will handle ethical reviews of its members. Nevertheless, non-compensation of ERC members may cause delays in ethical reviews because of the members' voluntary position or their other competing priorities. On the other hand, ERCs may also be under administrative "pressure" to take favorable decisions on proposals which may result in additional research funds, resources or other benefits for the institution. It is in situations such as these that the independence and integrity of the ERCs may be under suspicion or questioned. These problems, however, are not unique to developing countries; several US institutional review boards have been reported to have similar problems [12].

The functioning of any ERC involves costs. Training of the ERC members is an essential direct cost whose outcome would result in a more relevant and effective review. Equally important is the ERC members' continuing education and networking with other ERCs so that they are kept abreast of the more recent issues or controversies that may affect on their own skills and competencies to conduct ethical reviews.

Capacity-building for ERCs

There is a tremendous gap between the ethical review capabilities and competencies of ERCs of sponsors and that of developing countries. Currently, efforts are being carried out to reduce this gap. The WHO has facilitated the establishment of regional fora of ethical review committees for information exchange, development of national guidelines, standard operating procedures and educational activities for ERC members. There are currently four different fora – the Forum for Ethical Review Committees for Asia and the Pacific (FERCAP), Foro Latino Americano de Comités de Ética en Investigación en Salud [Latin American Forum of Ethics Committees in Health Research] (FLACEIS), Pan African Bioethics Initiative (PABIN) and Forum for Ethics Committees in the Confederation of Independent States (FECCIS). The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER) is an international cooperative endeavor

designed to facilitate the development of ethical review of medical research and is currently providing technical and financial support for these four fora. The Fogarty International Centre of the National Institute for Health has also sponsored bioethics training for faculties from developing countries.

Research and medical ethics training should be included as one of the capacity-building activities in all collaborative research as demonstrated by UNAIDS in relation to HIV vaccine research. These efforts can be among the most significant and permanent benefits that a study can provide its developing-country collaborators and could strengthen their relationships. Definitely, increased capacity of a developing country to conduct ethical reviews could lend to more effective collaborative research.

Ethical issues relative to informed consent

As increasingly more clinical trials are conducted in the developing countries, compliance with guidelines governing the conduct of these studies becomes more challenging. The issue of informed consent also gains additional focus as it is progressively applied in countries with culturally diverse settings and which are in a clearly disadvantaged position compared with its research sponsors.

Community research decisions

Several criteria are considered in the selection of a clinical research site, particularly for Phase II or III clinical studies. Obviously, research cannot be conducted in communities which will not accept and support it. Thus, one of these requirements should be the community's acceptance of and consent to research being conducted among its members. Agreement by local scientific collaborators to conduct the investigation is not enough. The acceptance, cooperation and support of local political leaders and health officials should be obtained at all levels – regional, provincial or state as well as at the district and village levels.

All international guidelines [3, 4, 8, 13] agree that the role of community consent is essential. But nowhere are these guidelines on community research decisions more explicitly detailed as in national or country guidance documents. For instance, studies which will be done among Aboriginals and Torres Strait Island communities require that a 'written documentation of consent from the communities' be obtained and to document the reasons where this was not possible [14]. Consultation is prescribed as an essential process in the research relationship and continuing research partnerships between researchers and the Maoris who will be involved in them [15]. The guidelines for the conduct of research in India

advocate the general principle of community agreement to all of its research, and specify that the principles of voluntariness and informed consent apply not only to individuals but also to communities [16]. The significance of community approval of research is so critical, that Weijer proposes "respect for communities" as the "fourth" ethical principle relevant to the ethics of research as stated in the Belmont Report [17].

But despite these provisions, there are no suggestions as to how community consent should be obtained. Literature reminds investigators that cultural sensitivity is essential in this process. Just what does this mean for researchers? Apparently, the first step is for researchers to have preliminary discussions with their local collaborators, who are assumed to be knowledgeable about and conversant with the local political hierarchy and structures, its decision-making dynamics, as well as the local culture and mores. They should be able to identify people's organizations or community leaders, possible collaborators, stakeholders or partners in the activity. The procedures and processes to obtain permission to conduct research may be dictated largely by the political protocol, community norms, traditions and practices. It may involve informal one-on-one face-to-face meetings, rites and ceremonies, group or village orientation sessions, an open forum, or structured discussion groups. The tones of these sessions may vary from that of information-giving, to consultation, especially if the research contains contentious issues, and even negotiations, to reach a consensus. It may necessitate only one visit or several return calls and discussions. It is a process which may not be hurried or shortened to meet the researchers' deadlines. The process of obtaining community consent should not be perceived as delaying or complicating the research process. The benefits which can be derived from such community consultation may make the difference between a smooth-sailing clinical research project against one which may be plagued with several conflicts and problems. The significance of the consultation process can be aptly illustrated in the following case study (Box 1).

Individual research decisions

Informed consent embodies the principle of respect for persons and their capacity to make choices. For consent to be valid and genuine, the following elements must be present: (1) complete and relevant information must be provided, (2) the prospective participant must have been given enough time to study her options and choices, (3) the information must be understood and (4) consent was voluntarily given. "Information sheet" will be the term used here to refer to the document used to obtain consent and "informed consent" is defined as a communication process between the researcher and the research participant that, according to CIOMS [13], "begins when the initial contact is made with the prospective subject and continues throughout the course of the study".

Box 1. Consultation as mechanism to ascertain study continuation

In the middle of a 5-year pediatric vaccine trial in Asia, the investigators were informed by the pharmaceutical company that the company had decided to cease clinical development of the vaccine under evaluation [36]. At that particular point, the trial was running smoothly with a monthly enrolment of 93% of the eligible population. The ethical question was: is it ethical to continue the trial knowing that the study vaccine did not have at that point any foreseeable commercial future?

As required, the investigators reported the situation to the appropriate national health authorities, who were for the continuation of the study. The institutional ethics committee, on the other hand, was for the discontinuation of the study, but nevertheless advised the research group to undertake the necessary community consultations.

Assured that safety was not the concern in stopping the clinical development of the trial product, the local stakeholders, i.e., the provincial and town-level health authorities, who considered themselves the "guardians of people's health", considered the risks and benefits provided by the trial to the community, and in appreciation of the evident benefits, voted for the continuation of the study. They took the responsibility of informing the political leadership of their recommendation and the reasons behind them. Upon their recommendations, the researchers also called for a consultation meeting with the midwives, to obtain their opinion and consensus on the issue. Apprehensions and concerns were expressed; explanation and further elucidation were offered by both the researchers and senior health officers. The consultation process concluded with the recommendation for the study to be continued despite the new information released by the vaccine company. The whole consultation process involved several meetings over a period of three months.

Equipped with the support of all the health staff of the trial site, the researchers and representatives of the provincial and town-level health authorities presented themselves to the ethics committee and formally requested the continuation of the pediatric trial.

Provision of complete and relevant information

For consent to be valid the participant should receive "complete, relevant and adequate" information [3]. International guidelines have been clear on, although not totally in agreement with,the information which should be included in these information sheets. Principle 22 of the DoH 2000 [8] states that:

> (I)n any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely given informed consent, preferably in writing. If the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed ([8], as amended in 2000).

Based on the above-mentioned principle, the DoH requires at least 8 essential elements on the information sheet. CIOMS, on the other hand, maintains that in addition to those stated above, 18 more pieces of information are necessary if an educated decision is to be made (Box 2). The International Conference of Harmonization-Good Clinical Practice (ICH-GCP) [18] guidelines agree with the elements proposed by DoH [8] and CIOMS [13], but listed a few more critical information elements which should be included in the information sheet.

With the volume of information required, the development of the information sheet to be used poses several challenges. When all the critical informed-consent elements are present, the information sheet may be as short as one page but could be as long as 15 pages. While it is easier to check the document's compliance with the provisions of the guidelines, its readability is a bit difficult to ascertain. The content of the information sheet is definitely novel, often complex and technical and could be intimidating to the educated, but more so to those who have less education and are unfamiliar with the concept of individual consent. The concepts of randomization, placebo or blinding may not have accurate equivalents in most languages and could thus be difficult to present in a manner ensuring its accurate translation. When Hochhauser [19] reviewed some 12 randomly selected information sheets used in clinical trials, he found out that they were difficult to read, contained several technical words, had a high word density per sentence and were not designed to encourage participants to read them.

As a way towards a more readable information sheet, some ERCs in the United States set an arbitrary grade-level requirement at which information sheets are supposed to be written [20]. Towards this end, some researchers use the Flesch-Kinkaid formula in Microsoft Word to determine whether the information sheet they are writing meets the readability criteria. However, there are arguments on the reliability of such measures [20, 21], making it imperative for researchers to explore other vehicles to ascertain readability.

Information sheets should be translated into the language or the dialect of the area where they are to be applied and used. The prescribed translation and back translation into English (the usual language of research protocols) may not be adequate. The translation may need to be subjected to the more rigorous exercise of pretesting or pilot-testing to ensure its comprehensibility [22]. This pre- and pilot-testing may complement other attempts to guarantee the tool's clarity and simplicity. For instance, the information sheet used in a pediatric Phase III vaccine trial in the Asia was pretested among health workers, mothers and grandmothers. The activity resulted in the validation of words/phrases used in the translation and the re-translation or rephrasing of other parts into the community's every-day language to be better understood. After the pretesting, the tool was further pilot-tested on mothers and caretakers. The exercise provided researchers with insights on how the information sheet was received – the reactions it

Box 2. Essential elements of informed consent according to the different guidelines

Declaration of Helsinki, 2000	CIOMS, 2002
1 Aims and methods of the study	1 Purpose of the study and procedures to be carried out by investigators
2 Sources of funding	2 Similar
3 Possible conflict of interest	3 Similar
4 Anticipated benefit	4 Direct benefit to participant; expected benefit to the community or to the society at large or contributions to scientific knowledge
5 Potential risks	5 Same including risks to partner or spouse
6 Discomforts	6 Similar
7 Right not to participate	7 Similar
8 Right to withdraw	8 Similar
	9 That individual is invited to participate in a research. reasons why the individual is suitable for research
	10 How research is different from routine medical care
	11 The expected duration of the individual's participation and the possibility of early termination of the trial or of the individual's participation in it;
	12 Compensation for participation
	13 That participants will be informed of findings related to their health status and the results of their study in general
	14 That the participants have the right to access their data on demand
	15 Any currently available alternative interventions or courses of treatment;
	16 Institutional affiliation of researchers
	17 Availability to the subjects of any products or interventions proven by the research to be safe and effective after they have completed their participation
	18 Provisions to ensure respect for the privacy of subjects and for the confidentiality of records in which subjects are identified;
	19 The limits, legal or other, to the investigators' ability to safeguard confidentiality, and the possible consequences of breaches of confidentiality;
	20 Whether the investigator is also the participant's physician
	21 The extent of the investigator's responsibility to provide medical services to the participant;
	22 Availability of free treatment, duration, organization providing treatment of specified research-related injuries and uncertainty of funds available for these
	23 How subjects or the subject's family or dependants will be compensated for disability or death resulting from such research-related injury
	24 Whether or not the right to compensation is legally guaranteed;
	25 That the protocol was approved or cleared by an ethical review committee

elicited, the questions it generated and the apprehensions and anxiety it created. The activity further resulted in the identification of procedures by which the information sheet could be applied.

Another concern in information sheet development is its layout and design. Since the document is usually long, it should be "attractive" in form and structure to encourage potential research participants to read and, if necessary, re-read it. There are no studies looking into the significance of layout or design as a stimulus to reading and comprehensibility of information sheets. However, one must recognize that the readability of any document is not only in its contents, but may also be affected by readers' literacy level and vision. Thus, researchers should make the effort to present these increasingly detailed and complex information sheets in a more attention-grabbing style. Consideration should be given to the size and type of printing font, whether the document should be presented in columns, the separation of the long document into Questions and Answers or sections or topics segregated by topic headings and the use of illustrations or boxes to highlight critical information.

Provision of enough time to study options and choices

The potential research participant needs to process an overwhelming amount of information to decide on his/her participation. It is essential that the procedures developed allow potential participants to have open access to the research promotional materials, facilitate continuous discussion and consultations if necessary and offer ample opportunity to thoroughly study and evaluate the information received. The international guidelines are very specific about this. CIOMS states that potential participants should be given the opportunity to ask questions; similarly, NBAC instructs researchers to devote time and effort to devising creative measures to facilitate comprehension of research information. These mechanisms should also be able to address previously identified problems, e.g., variability in the presentation of information and hesitancy of potential participants to ask questions [23].

Are there prescribed steps in obtaining consent? Is there a "preferable" way such that consent obtained is truly informed and voluntary? Cultural feasibility studies [24] or consultations with the community and the target population may be necessary in the development of informed consent. For instance, focus group discussions may result in the setting of systems to capture and identify target participants. These can inform investigators where and when discussions of the information sheets may be efficient, and what recruitment materials may be culturally acceptable. These advice-seeking sessions could also yield information on household decision-making dynamics – who makes decisions, who influences decisions – as a necessary input in targeting who should be invited into these recruitment processes.

Consultations could also result in the easy and well-supported solutions to problems which may deter implementation of trial activities.

The initial dissemination of information about the research activity could be presented to the general population and community through a massive information drive and campaign using both print and mass media and face-to-face interactive discussion groups - village meetings, open fora and the like [4, 25]. Giving lectures during community assemblies, meetings of professional or people's organizations, like medical societies and mothers' classes, may be efficient vehicles for a rapid diffusion of correct information and rapid feedback on people's perceptions of the activity. These introductory mechanisms are expected to generate some awareness about the activity and, it is hoped, create a demand for research-specific information among the target groups.

The information sheet is the commonly accepted mode of communicating research information. These sheets are provided to prospective research participants who are expected to read and understand them, prior to consenting to participate in a research activity. But simply reading the information sheet is not sufficient and satisfactory. Kuczewski and Marshall [26] believe that it should be a dynamic, interactive process, where the potential participant actively demands explanation and clarification, while the researcher provides facts, details and particulars about the trial. It must be conducted in a quiet, unhurried setting, where the participants feel free to read either by themselves or along with the investigator, review the information sheet and ask questions, clarifications or express anxieties and concerns. The potential participant must also be given the freedom to share the information sheet with the significant others in her/his social network or to seek their advice and opinions.

The assimilation of concepts presented in a multiple-paged information sheet may not be easy nor possible in one setting, particularly among less-educated participants. Thus, CIOMS proposes the adoption of a process where prospective subjects are informed orally about the research through repetition and explanation. This implies repeated discussions and consultations, and suggests that it does not take place only once, but rather over a period of time. If time permits, the researcher should allow the future participant to bring the information sheet home, to allow him the opportunity to engage in consultations with or seek the advice of the significant others in his social network. Some of the above-mentioned strategies were employed in clinical trials conducted in the Gambia [25], in rural Africa [27] and in other clinical research projects [28, 29]. The delivery of trial information in a pediatric vaccine trial in the Philippines was timed to coincide with the pregnant woman's/mother's clinic visits, i.e., from her prenatal visits until the newborn's first clinic visit for vaccination (Box 3) [30].

Brady [23] is wary about the ability of this text-based traditional consent procedure to capture the essence of informed consent for several rea-

Box 3. Informed consent in a pediatric vaccine trial in a South East Asian country

Firstly, trial brochures were placed at the health centers and hospitals, primarily for distribution to pregnant women who came for the prenatal check-ups; they were also distributed freely during mothers classes. This material introduced the vaccine trial to the target groups for recruitment. It triggered discussions between the pregnant women and mothers' whose children were already enrolled in the trial. It was the focus of deliberations between the pregnant woman and the health worker, whose advice and opinion about trial participation was also sought. Moreover, it was also used as a vehicle to introduce the EPI program to "new" mothers and reinforced or corrected the information held by the previous users of the service.

Dummy copies of the information sheet were provided to the newly delivered mothers either at the hospitals, during home *postpartum* visits or when the mothers or members of the family brought the newborn to the health center for their BCG vaccination. Upon their receipt of the document, the women were encouraged to read, share and if necessary discuss them with other household members.

A week or two prior to the child's expected clinic visit for the first DPT vaccination, trial staff visited the mothers at home to discuss the information sheet. The decision to make the home visit was gleaned from focus group discussions where child caretakers stated that: (1) they could not go nor stay long enough at the health centers for any reading or discussion of the information sheet because of other competing priorities in the home, i.e., other children, (2) they were shy and uncomfortable to ask questions in presence of other members of the community and (3) they want other household members, specifically the fathers, to participate in the discussion. The women added that although childhood vaccination was a decision mothers may make alone, the prospect of having a new, untested vaccine offered to the infants, is something that fathers should know about.

Depending on the outcome of the first home visit, mothers requested repeat visits at home or clarifications just before the child's vaccination.

sons. The extent and nature of the information presented may be culturally determined and thus obtaining a valid informed consent may be difficult. For instance, it is considered culturally inappropriate to disclose all the potential risks of research participation in Nigeria [4]. Japanese and Russian subjects demonstrate the attitude of "I put myself in your hands, doctors" [24], and thus may participate in clinical research without necessarily getting into the details of their participation. But these examples should not deter researchers from adhering to what are considered essential requirements to obtain valid consent. Therefore, the development of culturally responsive and creative techniques is essential. It is believed that research information could also be presented in culturally accepted media such as prose, stories, dramatization, street theater or ballad singers [3], which may be more suitable when research is conducted among less-literate populations [29, 31]. In more technologically advanced settings the use of network technologies, and employing such "edutainment" tools as illustrations, animation, etc., will greatly enhance informed-consent procedures.

Evaluating comprehension of information sheets

Another basic element for informed consent to be genuine and valid is that the research information should be understood. This implies that some sort of evaluation of the research participant's comprehension of the information sheet is made. The guidelines are not clear-cut about how this should be done and researchers have devised their own evaluation schemes. Fitzgerald et al. [32] required participants to pass some sort of examination using either true or false; Hochhauser [21] documented the use of multiple choice questions for such an evaluation, while others were asked to explain in their own words what they have understood from the information sheets [3]. Hochhauser [21] contends that there are problems inherent in the use of multiple-choice questions in evaluating comprehension and emphasizes that these are not sufficiently sensitive to detect true understanding and thus present limitations and complications in satisfying their element of valid consent [33].

Definitely, more experience and research are necessary to address several and varied issues relative to evaluating comprehension. Among others, these may include:

1. What information in the information sheet should be evaluated for comprehension?
2. When should this comprehension evaluation be done – soon after a discussion of the information sheet is done? Or should there be a gap from when the material was read or discussed by the researcher and the evaluation to be done?
3. How should evaluation of comprehension be done? Should prospective participants be given a pencil and paper test (despite its limitations)? Or should they be asked the questions verbally?
4. Should researchers set a "minimum level of understanding" scores? Who should set these? If the prospective participant fails to reach the minimum level of understanding, should s/he then be not included in the study? Who will make these decisions?
5. How will these evaluation scores be interpreted? Are these scores a function of the readability of the information sheet, the adequacy or deficiency of the informed consent process, a reflection of the reading ability of the research applicant, good or faulty recall of the prospective participant, or true comprehension?

While these issues may appear daunting, it does not justify not taking steps towards the need to find out what potential research participants really understand from the information sheets.

Access to products of research at the completion of the study

Among the controversial provisions in both the DoH and CIOMS guide-

lines is the access to the benefits of research by the participants, communities or countries. This issue touches the core of the conduct of research in developing countries since the non-availability of the proven intervention to the community from which the participants were drawn is regarded as exploitation. The NBAC report and the Nuffield document have presented exhaustive coverage and discussion of this issue based on expert reviews and discussions. Thus, it would be redundant if the same concerns are reiterated here. This section, however, will attempt to discuss the issue of what happens after the research is over in the case of a vaccine trial.

Vaccines are commodities which have wide public health applications. It takes a mean 15 years before a tried and tested candidate vaccine may be produced; it may take another 3–5 years before the vaccine may be licensed for large-scale use. Moreover, the whole clinical process may take several millions of dollars to undertake. When the proposed vaccine trial protocol is deliberated upon by an ERC for review, the questions in relation to the application of the relevant provisions in the DoH or CIOMs may include the following:

1. Is it the responsibility of the ERCs to be concerned about the future access to the vaccines at the completion of the trial?
2. Is the non-availability of a guarantee of access to the candidate vaccine after trial completion a reason for disapproving a vaccine trial protocol?

We propose that while ERCs are primarily concerned with the immediate risk-benefit ratios for the trial participants, they should look beyond and evaluate plans for providing the benefits of research, particularly those with wide public health impact, specifically when reviewing Phase III vaccine trial protocols. It is assumed here that the decision for bringing a vaccine to a Phase III clinical trial is based on favorable and significant immunogenicity information; in effect the prospects of the vaccine are good. This being the case, the ERCS may be justified in exercising their authority and evoking relevant provisions in the international guidelines when conducting the review. But this is not an easy task for the ERCs to do. They need to obtain answers from the research investigators to several questions prior to any deliberations and decisions they will need to take. Among the questions which should be addressed satisfactorily are:

1. What benefits should be provided to participants in a vaccine trial at its completion? If the vaccine trial had participants who received the placebo, what benefits should they receive at the completion of the vaccine trial? What will happen if the vaccine is not efficacious enough to warrant further production?
2. Is there anybody else who should receive the benefits of a vaccine trial at its completion? Who are these? What benefits should they receive?
3. What should be provided to vaccine trial participants, communities, etc., and should this be affected by the possible outcome of the vaccine trial?
4. What mechanisms should be employed to make these arrangements?

5. Since vaccines are public health commodities, who should make the above decisions?

These are just a few of several questions which investigators need to respond to in front of the ERCs. Moreover, the ERC should be able to appreciate what their responsibilities are in the implementation of the decisions as stated.

The nature of the above questions suggests that the issue of access to the products of vaccine trials needs careful planning, requires multi-level and wide consultations not only with the department of health officials, but also with other stakeholders and, more importantly, the community where the vaccine trial is proposed to be done (see Box 1).

In view of the above scenario, should the non-availability of arrangements for making available the products of a vaccine trial be a reason for disapproving a Phase III vaccine trial protocol? This is not an easy question to answer. In the case of vaccines, its long-term effects may not be readily evident during and immediately after the trial itself, and thus its provision without a long-term surveillance plan may have deleterious effects rather than the benefits expected [34, 35]. On the other hand, Bhutta [2] asserts that the benefits of research should not be viewed narrowly as simply the products of the research activity; these can be in the form of improvements in the health care system, training or technology transfer. This issue needs considerable reflection and deliberation – being very strict and stringent in complying with these provisions would definitely stop significant vaccine trials which may provide the necessary inputs for public health policies.

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Vaccine safety and adverse events: lessons learnt

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Introduction

The challenge for those who work in the field of vaccination is to collectively gather and present sound scientific evidence of the benefits and risks of vaccination, to recognise, understand and minimize the risks, and to define current vaccination policies as well as the future direction of vaccination. Convincing evidence needs to be presented to health professions, regulatory agencies, governments, and to parents who must decide whether their children should be vaccinated or not, despite uncertainties.

There have been several examples of recently identified serious adverse events being linked to vaccination, some of which were identified after licensure of the product, such as the occurrence of Bell's Palsy following vaccination with a new inactivated intranasal influenza vaccine [1, 2] and that of intussusception with rotavirus vaccination [3]. Some other purported claims of vaccine safety issues, such as that of a causal relationship between measles, mumps and rubella (MMR) vaccination and autism or inflammatory bowel disease or concerns about an "overload" effect of multiple childhood vaccinations on the immune system, have not been supported by scientific evidence but have had a major impact on vaccine acceptance and invited non-compliance [4–7]. These unsubstantiated threats to immunization programmes have had greatest impact in industrialized countries, due in part to a higher media coverage of claims of vaccination risks (whether or not such claims are correct), to the fact that many such claims involve medical conditions more prevalent or better recognized in industrialized countries (such as multiple sclerosis and autism), and to a lesser acceptance of small risks. As concerns in industrialized countries spread in the media or in the scientific literature, it is anticipated that they will have increasing impact in non-industrialized countries as well, potentially undermining vaccination coverage. Often an impact already exists in non-industrialized countries, albeit poorly quantified.

Although much progress has been made with respect to ensuring vaccine safety, with the further development of many additional vaccines and particularly those intended for poverty-related diseases, many safety issues will emerge, particularly in light of the technological needs of these newer vaccines and in light of the fact that some of these vaccines will mostly or even almost exclusively be used in non-industrialized countries. It is therefore of particular importance to acknowledge some of these issues and challenges and to consider the elements critical to overcome them so that safety issues do not later prevent the use and/or development of future vaccines.

In this article we: (1) briefly review the various elements of immunization safety, define the term "adverse events", and describe the steps taken to ensure and monitor vaccine safety; (2) highlight issues and challenges with respect to clinical trials and post-marketing surveillance in general terms and then more specifically with respect to new vaccines and vaccines intended for developing countries; and (3) propose some solutions or steps needed to improve the process, all of which are being promoted by WHO.

Immunization safety, adverse events, and steps taken to ensure and monitor vaccine safety

Scope of immunization safety

Immunization safety can be defined as the process to ensure and monitor the safety of all aspects of immunization, including vaccine quality, vaccine storage and handling, vaccine administration, disposal of sharps and management of waste [8]. In the past there have been dramatic examples of safety issues resulting from faulty production, such as the occurrence of tuberculosis following BCG vaccination in Lubeck in 1929–1930 [9], the episode of jaundice in military recruits following yellow fever vaccination [10] and the occurrence of polio following use of the inactivated Cutter polio vaccine in 1955 [11]. However, the risk of such incidents (most of which have been ascribed to a lack of scientific knowledge, gross negligence and lack of supervision and control in manufacturing procedures) [12], although not inexistent, has become more distant. Countries with properly functional national regulatory authorities have established independent laboratories to test vaccines and have obliged manufacturers to conform to strict standards of efficacy and safety. Detailed control procedures are now essential requirements of good vaccine manufacturing practice. In addition to ever improved and more sophisticated tests, vaccine regulations entail a number of other procedures to ensure safety, including the characterization of starting materials by supplier audits, cell banking, seed lot systems, compliance with the principles of good manufacturing practices, independent release of vaccines on a lot-by-lot basis by national regulatory authorities,

and enhanced pre- and post-marketing surveillance [13]. As a result, most of the risk with currently licensed vaccines, particularly in non-industrialized countries with limited health infrastructure, does not come from the vaccine itself but rather from a faulty usage, i.e., unsafe injections given with contaminated equipment resulting in a risk of transmission of blood-borne pathogens (HIV, Hepatitis B and Hepatitis C) following syringe reuse [14] or toxic shock following contamination of reconstituted multidose vaccine vials kept over the recommended period of time [15] or intoxication as a result of reconstitution of lyophilized vaccine with improper diluent or inadvertent inoculation of other drugs. Such incidents have included administration of pancuronium bromide, selenium, and insulin among other substances and have unfortunately resulted in deaths [16].

Innovations in safe vaccine administration such as auto-disable syringes that prevent reuse and other pre-filled devices with similar advantages have now been widely introduced into immunization programs in developing countries and contributed to tremendously reducing the previous risks of unsafe injections [8, 17, 18]. Further efforts are ongoing to prevent infection of health workers through accidental needle stick injuries and to avoid risk to communities through proper sharp waste disposal [8]. Vaccines are now mostly "bundled" with auto-disable syringes and disposal boxes, thus ensuring adequate supplies of equipment to maintain safe injection practices. Developments in needle-free technology are also taking place, particularly focusing on jet injectors using disposable standard single-dose cartridges; multidose jet injectors previously being considered for use in immunization programs are currently faced with virtually insurmountable challenges with respect to demonstrating safety [personal communication Martin Friede, WHO]. Greater attention than ever before is being paid in non-industrialized countries to safe vaccine administration and monitoring and management of adverse events.

Adverse events following immunization

For monitoring purposes, an adverse event following immunization (AEFI) can be defined as an untoward medical event temporally associated with immunization that causes concern and is believed to be caused by immunization but might not be actually caused by the vaccine or the immunization process. This includes true adverse reactions caused by the vaccine's inherent properties, possible programmatic errors caused by faulty vaccine preparation, handling or administration (e.g., use of non-sterile equipment, use of improper diluent for reconstitution as mentioned above) and coincidental events (i.e., an event that happens after but is not caused by immunization). Due to the large number of doses administered, there are indeed many coincidental temporal associations between medical events and vaccine administration. In addition, the fear of injection may result in anxiety

reactions, including fainting or other psychogenic illness that are related to the injection itself rather than to the vaccine.

In each individual case, and with few exceptions, particularly for live attenuated vaccines for which the vaccine strain can be sequenced (e.g., yellow fever vaccine-related multi-organ failure [19], mumps vaccine-derived meningitis [20]), there is no pathognomonic syndrome and no clear markers of etiology and it is often difficult to demonstrate or rule out a definite relationship between a vaccine and a particular medical condition.

True vaccine reactions are generally mild. More serious complications are rare. Severe reactions resulting in death or permanent damage are extremely rare. By far not every complication that follows the administration of a vaccine is linked to its antigenic components. Problems can also be attributed to a carrier or excipient rather than to a primary constituent. Vaccines, especially whole bacterial vaccines, often contain pyrogens that cause fever by release of chemicals from macrophages [21]. Adjuvants enhance the antibody response to vaccines but aluminium hydroxide and phosphate may induce local reactions such as induration and swelling. For many years hypersensitivity reactions to measles-containing vaccines were thought to be due to egg protein as the vaccine was produced in chick embryo tissue culture. Yet studies using sensitive techniques have not detected residual egg protein and children who had hypersensitivity reactions after receipt of measles-containing vaccines have been demonstrated to react to the gelatine stabilizer rather than to egg proteins [21–24]. As a matter of fact any vaccine component could arguably be subjected to close scrutiny for its potential capacity to produce serious reactions.

Avoidable adverse events are associated with faulty production (e.g., bacterial or viral contamination or insufficient inactivation, presence of foreign toxin) or programme mistakes mentioned above . A useful and more complete description of adverse events and their causation is provided by Halsey et al. [21]. More information on the occurrence of specific reactions can also be found in WHO reference documents that are periodically updated [25, 26].

Vaccines are said to be victims of their own success. As immunization programs reduce target diseases, adverse events inevitably gain in importance. Negative publicity also tends to put the risk of immunization out of perspective and one loses the sense that most vaccine-preventable diseases still occur and some could re-emerge. The media are prone to propagate vaccine safety issues, and the public to go along with them, at the expense of the recognition of the vaccines' benefits. Anything that occurs after vaccination is likely to be called an adverse event. The saga of the purported risk of autism following MMR vaccination in the UK is an unfortunate example of this [4–6]. Vaccine safety concerns also arise in non-industrialized countries and may be a product of special characteristics of the health service. Fears in India about a potential contraceptive effect of the oral poliovirus vaccine (OPV), possibly related to participation of health work-

ers from routine family planning services in mass OPV campaigns, is such an example [27]. More recent allegations of a contamination of OPV with hormones in northern Nigeria which gravely threatened the global polio eradication effort appeared to have more political motives [28]. False assumptions regarding causality are likely to occur for illnesses without a clearly defined etiology or pathogenesis, allowing for speculation about the role of vaccines or their constituents. In several instances, individual investigators have made observations and speculated way beyond their data to imply causal relationships between vaccines and specific medical conditions.

Steps taken to ensure and monitor vaccine safety

Pre-licensure testing of vaccines includes pre-clinical testing in appropriate animal models as well as three phases of studies in human subjects. Phase I safety and immunogenicity testing involves a small number of low-risk individuals (usually healthy immuno-competent adults) who receive the vaccine to determine tolerability. Phase II dose-ranging studies may involve hundreds of individuals to determine a safe and immunogenic dose. The design of phase II studies may vary and include controls [29]. Phase III studies are the more classic, randomised, double-blind, controlled trials involving thousands to ten thousands of more heterogeneous individuals. These studies are designed to address clinical efficacy in disease prevention, provide further safety information, and serve as the final gate-keepers prior to vaccine introduction to the public market [30]. There are limitations, however, to what we can learn from phase III trials. The nature of pre-licensure trials limits the number of subjects studied and thus limits statistical power. Pre-licensure studies can identify events with a magnitude from 1 in 1,000 to 1 in 10,000. Bigger studies to detect more rare conditions would be conducted at prohibitive cost. According to a mathematical approximation, the rule of three indicates that to detect a given risk you must enrol 3 times more exposed subjects, e.g., 3,000 subjects to detect a risk of 1/1,000 doses. Phase III trials are therefore designed for the detection of common and acute reactions and not generally designed to detect uncommon reactions or reactions with vague or delayed onset. In addition, phase III studies have limitations in their ability to detect interactions and to examine outcomes in special populations and in the general population. As well, difficulties with manufacturing biological products may potentially lead to the inadvertent release of vaccine lots that are not fully protective or that may be more reactogenic and hence are beyond the possibility of detection at the pre-licensure stage.

All of the above points emphasize the importance of post-marketing surveillance for AEFI (including true vaccine reactions). For newer vaccines it is becoming common practice for the National Regulatory Authority (NRA) to require manufacturers to perform post-marketing surveillance or

post-market studies to further assess the safety and effectiveness profile of a vaccine. Although it is sometimes appropriate to evaluate new vaccines with randomized control trials after licensure, it is not always ethically acceptable, logistically feasible or even scientifically appropriate to use randomized control trials. For these reasons observational study designs are most commonly used to evaluate the safety and efficacy of a vaccine in the post-licensure stage. Such studies are observational *versus* experimental in nature as the investigator does not allocate subjects to receive alternative vaccines (or interventions) according to any deliberate plan.

The purpose of post-marketing surveillance is (1) to detect, correct and prevent program errors, (2) identify problems with vaccine lots or brands, (3) estimate rates of occurrence of AEFI in the local population, (4) identify signals for unexpected adverse events and generate hypotheses that must be confirmed by controlled studies or laboratory work, and (5) help formulate and adjust contraindications, the risk-benefit equation, and information provided to the patient. Effective post-marketing surveillance in turn will help prevent false blame attributed to coincidental events and maintain confidence in the program.

In the vast majority of countries where it exists, post-marketing surveillance is performed by spontaneous reporting or so-called passive surveillance using various reporting schemes whereby reporting of cases thought to be due to or that could be due to vaccination is expected. The reporting is either linked to or separated from that of adverse drug reactions monitoring. At times reporting is mandatory; however, with a few country exceptions, mandatory reporting seems to provide a weak (if any) incentive to report. Extensive information on this topic and guidelines for reporting of AEFI are available on the WHO website (www.who.int/immunization_safety_en/) [31, 32]. Very few countries enjoy some specific sentinel active surveillance system or specifically targeted active surveillance such as those set up through paediatric hospitals or those set up for mass vaccination campaigns [33, 34].

Issues and challenges

Limits of clinical trials

Because of their relatively large size, phase III trials offer a unique opportunity to evaluate vaccine safety. In past years, however, the approach taken in phase III trials was often to focus on documenting common reactions of mild severity such as fever and pain or erythema at the injection site. Often these trials measured adverse events only in a (at times quite small) subsample of the trial population. Some trials even failed to conduct surveillance for adverse events [30]. In addition, the duration of follow-up is at times too limited to capture some potentially serious AEFIs.

Fortunately, several changes have occurred in the way safety is documented. First, it is recognized that not all adverse events can be easily predicted. For example, intussusception following oral receipt of live rhesus reassortant rotavirus vaccine was not an expected side-effect of the vaccine [3]. Therefore it is inappropriate to limit the focus in phase III trials to those events that can be readily predicted. As well, it is no longer acceptable to target surveillance only to common adverse events or to place only a subsample of participants under surveillance for adverse events. Newer-generation live oral rotavirus vaccines have been or are being tested in phase III trials that are large enough to detect an attributable incidence of intussusception of approximately 1 case in 10,000 vaccine recipients [35].

Ionnanidis and Lau [36] have highlighted that the quality and quantity of safety reporting in randomized trials were often inadequate. The WHO vaccine trial registry, mostly concerned with trials implemented in developing countries, was used to assess whether and how investigators are monitoring AEFIs in vaccine trials [37]. As of August 1999, when analyzed by Val Mayans et al., the vaccine trial registry included 68 trials completed or in progress concerning a wide spectrum of infectious agents and diseases. This review highlighted that AEFIs were mentioned in only 69% of phase III and 42% of phase IV trials; few of the trials provided details on the types of AEFI monitored. The good news was the positive trend toward an improvement over time; 54% of 26 trials started in 1987–1993 mentioned AEFIs, compared with 81% of 42 trials started in 1994–99.

Investigators need to place emphasis on AEFI monitoring methods and include results in any publication along with vaccine efficacy methods and results and it is important that causality assessments be carried out for serious adverse events in clinical trials.

Furthermore, editors and reviewers need to be aware that AEFI data, even when negative and reassuring, are essential information in publications of vaccine trials. Yet there have been many situations where safety information, although it exists and would be critical in the interpretation of other data, is not available to those investigating a particular safety problem.

Limits of post-marketing surveillance

Well-known limitations of post-marketing surveillance include: incomplete data, lack of verification of reported diagnosis, faulty numerator and denominator data, lack of standardization and reporting bias. Post-marketing surveillance data cannot be used for calculating true relative risks or true incidence rates. Causal associations between a vaccine and an adverse event can rarely be determined by specific tests such as identifying a vaccine agent in the affected tissue of patients. In the absence of such data and with the limitations of surveillance data mentioned above, epidemiological

studies have to be used to determine if the risk of the condition is increased in vaccinated *versus* unvaccinated individuals. Yet common mistakes include assuming a causal relationship based on a temporal association only or on a series of affected patients. Because vaccines are administered to large segments of the population it is not surprising that a substantial proportion of cases of a particular disease would occur within a given window of time after vaccination, yet surveillance only focuses on those vaccinated individuals expressing a given condition. Determination of the actual risk and causality in epidemiological terms would need consideration of the entire picture and calculation of relative risks, i.e., risk in vaccinated populations/risk in unvaccinated populations.

Fortunately, post-marketing surveillance capability is improving; more countries now have AEFI monitoring systems and more importance is attached to the reporting of suspected links between vaccination and adverse events (termed "signals") and their further investigation. Despite much improvement in the global capacity to monitor adverse events, much progress remains to be made, particularly in developing countries, including addressing a common weakness of limitations in the ability to properly analyze and interpret the data.

Careful studies have demonstrated that many hypothesized (on the basis of surveillance data) causal associations between vaccines and adverse events were not substantiated. Surveillance and signal generation, particularly fishing expeditions in large databases, have the potential to identify spurious associations and generate false hypotheses. Thus one should exert caution before raising a signal and highlighting issues to the general public. It is also fundamental for researchers to differentiate between hypothesis generation and hypothesis testing. Although most of the purported associations have been properly investigated and health risks ruled out, this has created a huge amount of work, public attention and avoidance of vaccination. Because of the many assertions made about vaccine safety and their potential to unduly threaten public confidence in vaccines, there is a need for quick hypothesis testing and for adequate tools for hypothesis testing to be available proactively. It has proven essential that suitably controlled studies be undertaken and completed rapidly when such assertions arise.

Investigating hypotheses

Study designs to investigate signals include case-control studies and cohort studies. The latter are better suited for outcomes that occur shortly after vaccination and where the outcome under study occurs frequently. When an outcome is rare or occurs with a long interval after vaccination, it is usually more feasible to evaluate the vaccine by using case-control designs where cases and controls who did not develop the disease outcome are compared

for earlier receipt of vaccination. A more recently proposed design particularly suitable to situations in which coverage is high is referred to as self-controlled case-series analysis in which the incidence of adverse events of interest is compared within two windows of time for the same subject, i.e., a window of vulnerability (the post-dosing interval in which the adverse event is postulated to occur) and a window of invulnerability (another post-dosing interval in which the adverse event is postulated not to occur) [38]. This design was used in particular in the evaluation of intussusception following receipt of the live rotavirus-reassortant vaccine [3] as well as in an evaluation of the potential occurrence of intussusception following receipt of OPV during the same period [39, 40].

Special large linked computerized databases such as the one established by the US Centers for Disease Control and Prevention's Vaccine Safety Datalink [41] or the General Practice Research Database in the UK have a great potential to facilitate the investigation of safety issues [42]. These databases link histories of receipt and non-receipt of vaccines in a defined population with comprehensive records of treatments and hospitalizations. Demographic and socio-economic data are also collected. These databases enable rapid controlled analysis and are getting more and more commonly used in industrialized countries but are still virtually absent from developing countries with the rare exception such as Viet Nam [43].

Only a few countries have the capacity and tools to complete hypothesis testing and international collaboration is therefore vital. The process adopted for the investigation of a potential link between oral polio vaccination and intussuception is a model [39, 40]. International collaboration in hypothesis testing and independent scientific reviews of studies enabled the hypothesis of a causal relationship to be rejected, thereby avoiding the potential damage to vaccine acceptance and the polio eradication objectives.

Specific issues of new vaccines and vaccines for poverty-related diseases

While exciting, the impending flood of new and potential products and technologies must be accompanied by a political commitment to secure the necessary resources to keep up the evaluation of vaccines [44]. Although a number of additional issues and challenges are not exclusively related to vaccines for poverty-related diseases, some need particular emphasis because of the nature of the vaccines under development or the characteristics of the countries where these vaccines would be primarily used. A number of these issues are discussed below.

The first issue concerns adjuvants. During the past decade, a large number of novel adjuvants have been developed and evaluated clinically, several of which are already contained in licensed vaccines. Others are likely to

be available in coming years, including oil-based emulsions such as MF59 and Montanide ISA 720, immunostimulators such as monophosphoryl lipid A, CpG oligonucleotides, saponins such as QS21, and mucosal adjuvants based on bacterial exotoxins that have been developed for nasal and oral delivery. A number of these adjuvants will have a bearing on the scientific development, efficacy, safety and quality of new vaccines developed for HIV/AIDS, tuberculosis, malaria, leishmaniasis and other conditions. Safety considerations require a thorough understanding of the effects of adjuvants on the immune response and related mechanisms. Yet there is little understanding of the important field of adjuvant safety [45]. Since adjuvants have their own pharmacological properties, which might affect both the immunogenicity and the safety of vaccines, assessment of adjuvant safety in itself is essential. Adverse events attributable to adjuvants need to be documented and reviewed, and the information made available. Animal models have limitations in predicting adjuvant safety. Validated animal models for adjuvant safety testing do not exist, yet they are required for future vaccine research and development. Short-term and long-term safety evaluation and prediction are important, as is the evaluation of the pharmacokinetics of the adjuvant alone. Safety testing of new adjuvants, once combined with a vaccine, necessitates improved and consistent standards for safety monitoring boards, post-licensure signal generation and evaluation, and surveillance of adverse immunological events. This includes unexpected safety issues. Phase IV studies should accommodate adverse reactions that cannot be anticipated from the biological actions of the vaccine and its adjuvant, including the rare and unusual adverse reactions that are not detected in animal work or in small pre-licensure human studies. All this requires a new clinical, scientific and regulatory approach, with attention to the short- and long-term safety of adjuvants [45]. Since many of the new adjuvants are likely to be used in vaccines for conditions endemic in developing countries, scientists from those countries should be aware of these issues and involved in related discussions.

Another issue is that a surveillance focus and capacity must then be available in countries where vaccines are newly introduced and conversely the choice of where to introduce new vaccines first may have to take into consideration the vaccine-preventable disease and AEFI monitoring capacity of the countries. The case of dengue vaccine development illustrates the point. Dengue virus is a flavivirus with four serotypes and type-specific protective immunity. At least eight vaccines against dengue virus infection are under development. Sensitization to severe dengue illness through pre-existing immunity is a serious safety concern for vaccination. Such sensitization has been observed in the course of sequential dengue epidemics by different virus strains in Cuba [46]. From these and other data, the relative risk of severe disease following secondary heterotypic infection has been estimated to be 15 to 80. While host genetic as well as viral virulence factors might contribute to severe dengue hemorrhagic fever, viral load (infectious

pressure) and concentration of antibody also appear to play a role. One of the implications of sensitization to dengue is that dengue vaccines need to elicit a balanced immunity to all four dengue serotypes. The 4–5-year follow-up of a tetravalent vaccine currently under clinical evaluation has not revealed a risk of severe disease among vaccine recipients, but the accrued data are still relatively small. It will be important to study the early immune response, its kinetics, and the relationship of these to vaccine-induced memory. Long-term AEFI follow-up is required. The sero-epidemiology of dengue fever should be determined in order to evaluate dengue vaccine safety before and after general introduction of the vaccine, with special attention to the impact of herd immunity [45, 47, 48].

In the past, vaccines that were developed in the industrialized world were used in the country of manufacture and then gradually reached the developing world, and regulatory authorities in the developing world based their decisions on those taken in the industrialized world. The current situation is that there are vaccines produced in Europe which are only required in developing countries. In view of this situation, the European Medicine Agency (EMEA) has changed its regulations with regard to marketing authorizations for vaccines not marketed in the European Union within 3 years of registration; the license will cease to be valid, as opposed to renewal of the license every 5 years, regardless of the market where the product was placed. Discussions between EMEA and WHO resulted in EMEA article 58. This article states that "the Agency may give a *scientific opinion*, in the context of cooperation with WHO, for evaluation of certain medicinal products for human use intended exclusively for markets outside the Community…" [49]. The process will entail the same thorough assessment as is undertaken for a license, but will end in an opinion on the quality, safety and efficacy of a product, as opposed to a marketing authorization. There are, however, a number of issues which are raised by this new procedure. A key issue is the acceptability of the new procedure in countries that will be recipients of this opinion. This is the subject of ongoing consultation.

Another issue to be considered is that of population risk *versus* individual risk which is particularly critical in developing countries where financial constraints may prevent achievement of a high vaccine coverage. An example of this is in the context of the congenital rubella syndrome. When children are immunized on a large scale against rubella but without reaching coverage levels high enough to interrupt transmission, individuals are less likely to encounter the disease at an early age and gain natural protection before reaching reproductive age. If no consideration is given to immunization of females prior to reproductive age, one runs the risk of an increase in infection during pregnancy and hence of a potential temporary increase in congenital rubella syndrome cases. This risk was unfortunately proven to be real in Greece [50].

Although novel vaccine production methods and delivery technologies could theoretically result in safer, more efficacious, more convenient and

potentially cheaper-to-produce vaccines with the added benefit of avoiding injections and therefore improving safety, new vaccines and vaccine delivery technologies are not necessarily safer. This is well illustrated by the recent examples of two non-parenteral vaccines, an oral vaccine against rotavirus and an intranasal influenza vaccine with heat-labile *Escherichia coli* enterotoxin as adjuvant, both of which were withdrawn soon after marketing as a result of adverse reactions: Bell's palsy in the case of the influenza vaccine and intussusception in the case of the rotavirus vaccine already mentioned [2, 3].

Although in the pre-licensure trial of the influenza vaccine no case of Bell's palsy had been reported, during the seven-month period after introduction on the Swiss market (i.e., October 2000 through April 2001) 46 cases of Bell's palsy were reported. A subsequent case-control study estimated that the risk of Bell's palsy in vaccinated as compared with unvaccinated persons was 84 (adjusted odds ratio; 95% CI 20-352) with a relative risk of 19 and an excess risk of 13 per 10,000 vaccinees under the most conservative assumptions. Of the identified 91 intranasally vaccinated patients with Bell's palsy, only 21% had an onset interval of 1 to 30 days after vaccination, i.e., within the duration of follow-up in the context of the pre-licensure trials [2].

Five cases of intussusception were seen among 10,054 infants who received the rotavirus vaccine compared with 1 case among the 4,633 infants in the placebo group [3]. In a case-control study, the incidence rate ratio was estimated at 29 (95% CI 16-54) within two weeks of the first dose. The second dose increased the risk more modestly. Overall there was one case of intussusception attributable to the vaccine for every 4,670 to 9,474 infants vaccinated.

A number of lessons can be learnt from these vaccine withdrawals, some of which have been highlighted by Li Wan Po [51]: first, all vaccines carry some risk of an adverse effect that needs to be balanced against their potential benefits and, second, for diseases for which there are current safe alternatives available, post-marketing surveillance should be intense given the limited pre-marketing testing of some vaccines. In addition, it is important to have the right balance between delaying the benefits and safety concerns. Although the assessment should be country-specific, it is clearly based on the rotavirus experience that the risk/benefit ratio or perception of risk in industrialized countries may unduly influence the decision-making process in developing countries where the ratio may actually be very different.

The growing number of vaccines and limited opportunities for contact with the health care system imposes considerable pressure to combine schedules and antigens within a vaccine to minimize vaccine-related visits as well as the number of injections required. Although on the one hand multivalent vaccines have great advantages such as reduced administration costs and increased coverage, the increased complexity of the antigen mix-

ture makes the determination of adverse event causality very difficult and complicates the assessments of efficacy and safety. As well, the wide-reaching nature of immunization programs in developing countries and limited ability to deliver other preventive and curative health interventions make it tempting and efficient to explore the potential to deliver other health interventions concomitantly with vaccination. Some of these interventions, such as the delivery of intermittent treatment against malaria or antihelmintics, have the potential to interfere both negatively and positively with the immune response and this potential interaction should be assessed.

A major challenge of vaccines against poverty-related diseases is the high prevalence of malnourished children and immunocompromised populations in those countries where the vaccines would be introduced. This may have major implications in terms of safety and should be addressed as early as possible in the life cycle of the vaccine. For example, there is still a paucity of population-based data on the effectiveness of the current live BCG vaccine in preventing severe tuberculosis in HIV-positive infants and on its safety. It has been recommended that population-based studies should be undertaken to determine the efficacy and safety of BCG and other vaccines in both HIV-negative and HIV-positive children where there is a high endemic rate of tuberculosis [52].

For those involved with vaccines aimed at poverty reduction and at prevention of diseases specific to developing countries, it is important to be aware of the prequalification process for vaccines to be provided through the UN system, a needed reality particularly for those countries too poor to afford the vaccines through other purchasing mechanisms. WHO provides a service to UNICEF and other UN agencies that purchase vaccines, to determine the acceptability in principle of vaccines from different sources for supply to these agencies (www.who.int/immunization_safety/en/). This is done through an established procedure for the initial evaluation of candidate vaccines. Reassessment at regular intervals ensures the continued quality of vaccines currently being supplied. The goal is to ensure that vaccines used in immunization programmes are safe and effective and that they meet the specific needs of the program, reflected by the tender specifications: i.e., potency, thermostability, presentation, labelling, shipping conditions, etc. The process relies on the NRA of the producing country, understanding of production process and quality control method, production consistency ensured through compliance with good manufacturing practices, random testing for compliance with specifications, and monitoring of complaints from the field [53]. As a result of this evaluation process, WHO issues a regularly-updated list of vaccines which have successfully fulfilled all the established conditions and can thus be taken into consideration for purchase by UN agencies. This list is public and available on the web and can also be used by countries procuring vaccines directly. The fact that a given vaccine is not included in the WHO list of prequalified vaccine products does not necessarily mean that the vaccine would not meet WHO

requirements. It may only mean that it has not undergone the prequalifying process.

One challenge facing the prequalification process is that of vaccines produced in developing countries which, even though they are submitted to the same scrutiny as those produced in the western world and manufactured in top world class facilities, tend to be faced with distrust and the erroneous belief that they are of suboptimal quality. Yet these vaccines, including existing vaccines such as DPT, measles-containing vaccines or hepatitis B vaccines, are of critical importance globally and specifically for developing countries since the global supply, and particularly that procured through the UN system, heavily relies on them.

Solutions

WHO has a critical role to play in ensuring vaccine safety at the global level. First, it has an advocacy and normative role and, second, a role in determining and facilitating sharing of information on the safety profile of vaccines based on the best scientific evidence. Most of all, WHO is providing technical support to help strengthen the capacity of member states, particularly in the non-industrialized countries, to monitor and manage vaccine safety issues. The following provides a description of four major activities/tools that WHO has been involved with in support of the above roles.

Capacity building for the NRA and ability to implement the post-marketing surveillance function

Assessment of NRAs began several years ago with the objective of gaining a clearer idea of how well NRAs were performing and ultimately strengthening them. Initially, the NRA assessment was a capacity-building exercise; it is now linked to prequalification of vaccines. In order for a vaccine to be accepted for prequalification, the NRA of the producing country must have been assessed, with positive results in terms of performance of defined core functions. In view of the link with the prequalification process, there is a need to become more stringent with the criteria that NRAs have to meet and indicators of performance in the various NRA functions have been periodically updated [54, 55]. The review also results in a developmental plan to address weaknesses with support from the Global Training Network (GTN). In June 1996, the GTN was established by WHO to provide educational resources to staff involved in vaccine regulations, programs and production throughout the world. The network consists of 12 training centres which offer instruction in priority areas using approved syllabi and standardized materials [56]. The network provides NRAs, National Control Laboratories, vaccine producers meeting specific requirements and nation-

al immunization program staff with training in the following courses: Good Manufacturing Practice; Laboratory Quality Systems; Quality Control Testing; DTP Vaccine Production (for regulatory staff); Regulation of Vaccines; Laboratory Animal Science and Husbandry; Surveillance for Adverse Events Following Immunization; and Lot Release and Laboratory Access [56]. Additional activities to strengthen country capacity include the provision of guidelines and various resource materials, all of which can be found on the WHO safety website at www.who.int/immunization_safety/en/.

Guidelines on clinical evaluation of vaccines: regulatory expectations

Guidelines have been developed and published by WHO to assist regulatory authorities and vaccine manufacturers with the clinical evaluation of vaccines; the document can also be useful for clinical researchers and investigators [57]. These guidelines emphasize that randomized studies must have sufficient power to provide reliable rates of common (>1/100 and <1/10) adverse events, and to detect less common but not necessarily very rare (<10,000) adverse events and provide specific recommendations on post-marketing surveillance and phase IV studies.

Guidelines for preclinical evaluation with a focus on safety have also been developed [58].

Standard definitions

The diversity of case definitions and the variability of assessment methods and cut-off points used in vaccine trials and reports from post-marketing surveillance are huge and concern all types of adverse events or reactions. This was illustrated by Bonhoeffer et al. who reviewed published results from 10 large prospective trials [59]. Such differences in AEFI definitions and categorization limit meaningful comparison or meta-analyses and result in a considerable loss of scientific information. A key element in making different study results comparable is a mutual understanding of the adverse event under study. The Brighton Collaboration was launched in the Fall of 2000 as a global collaboration to facilitate the development, evaluation, and dissemination of high-quality information about the safety of human vaccines through the development of standardized case definitions and monitoring guidelines for AEFI. Voluntary working groups comprised of participants from public health, academia, regulatory organizations and industry develop standardized case definitions and guidelines for data collection, analysis, and presentation through literature reviews and consensus formation [59]. A larger reference group comprising over 500 experts from over 60 countries is offered to review draft documents prior to finalization.

The first six Brighton case definitions and corresponding guidelines to be finalized were for fever, generalized convulsive seizure, hypotonic-hyporesponsive episode, intussusception, nodule at injection site, and persistent crying [60–65]. Sixteen additional definitions are at various stages of development, i.e., abscess and cellulitis at injection site, anaphylaxis, encephalitis, fatiguing illness including chronic fatigue syndrome, local reactions including swelling and induration at injection site, aseptic meningitis, rash, and sudden unexpected death syndrome including sudden infant death syndrome. Altogether the aim is to develop up to 50–100 definitions. More information can be accessed at the Brighton Collaboration web site brightoncollaboration.org. It is important that these definitions are suitable for all settings and that more and more scientists (regulatory authorities, academicians and so on) from developing countries participate in the Brighton Collaboration process.

Global Advisory Committee on Vaccine Safety (GACVS)

As highlighted above, rumours of vaccine risks should be dealt with and investigated urgently and decisively if the potential negative impact on vaccine acceptance is to be prevented or necessary modification of programs to be implemented. Appropriate systems for response are required in every country and international collaboration is needed to deal with safety issues of international or regional concern. National advisory committees or review panels exist in a number of countries to specifically review safety issues. These committees, however, have essentially a national mandate and perspective and may differ in opinion based on a number of factors. Considering its role to serve all countries, the WHO established a Global Advisory Committee on Vaccine Safety in 1999 to provide independent scientific assessments of serious vaccine safety issues of potential global importance [66, 67]. The committee also assesses the implications of vaccine safety issues for practice worldwide and for WHO policies. In doing so, the GACVS often draws on the advice, experience and analysis of outside experts. The committee is particularly useful in helping WHO get the best recommendation and assessment of risks in situations of doubt or uncertainty. One of the first activities of the GACVS was to determine a set of criteria according to which the causality of adverse events following immunization could be judged [68]. The committee has reviewed many safety issues to date, including: macrophagic myofasciitis and aluminium-containing vaccines; health effects of thiomersal-containing vaccines; autoimmune diseases and vaccines; child mortality following routine infant immunization; safety of yellow fever vaccine; risks following immunization in HIV-infected children; safety of BCG vaccine in immunocompromised persons; MMR and autism; multiple sclerosis and hepatitis B vaccination; purported non-specific adverse effects attributed to DTP vaccines in infants; acute

lymphatic leukaemia and hepatitis B vaccination; oculo-respiratory syndrome following influenza vaccination; Bell's palsy following vaccination with an inactivated intranasal flu vaccine; and influenza vaccination of women during pregnancy. The outcome of the deliberations of the committee on these and other issues is reported routinely in the Weekly Epidemiological Record [45, 52, 69, 70] and is to be found at http://www.who.int/vaccine_safety/en/.

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Sequential stages of clinical trials and overview of issues to be considered

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Introduction

We live in an exciting era in which the fruits of molecular biology and biotechnology are yielding a profusion of new and improved candidate vaccines, delivery systems, and adjuvants that have the potential to control many of the infectious disease scourges of the developing world. Indeed, it has been estimated recently that there are over 350 vaccine candidates currently under development against nearly 100 infectious diseases [1].

Discovery of a new vaccine candidate and demonstration of its safety, immunogenicity, and protectivity in animal models are, however, only the first steps toward licensure and introduction of the vaccine into public health practice. The longest phase of development for most vaccines before licensure, and arguably the most uncertain, laborious, and expensive phase, comprises clinical testing of the vaccine in humans [2]. At a minimum, this clinical testing must demonstrate the vaccine to be acceptably safe and suitably protective in the population that will ultimately be targeted for the vaccine in public health practice [3].

Over the years, regulatory agencies have adopted a standard paradigm for the manner in which new vaccines are tested in humans. A key feature of this paradigm is the phased fashion in which the testing occurs. This chapter describes the phases of vaccine evaluations in humans, including several considerations for the phased testing of vaccines in developing countries.

Rationale for phased testing of vaccines in humans

Vaccines have had a remarkable track record of safety. Nevertheless, even when manufactured flawlessly, some vaccines have caused serious side-effects [4]. The mechanisms for these side-effects are diverse and are sometimes related to such problems as vaccine-induced immunopathology, as occurred with early-generation measles and respiratory syncytial virus vaccines [5, 6]. At times the mechanism may be obscure, as was the case for

intussusception induced by quadrivalent rhesus reassortant vaccine against rotavirus diarrhea [7, 8]. Whatever the mechanism, the point to be noted is that such severe side-effects are not always predictable. To minimize potential injuries to subjects caused by vaccine side-effects during re-licensure trials, vaccines are tested in a phased manner. In this phasing, early evaluations are conducted in small numbers of subjects, so that if reactions are observed, they will affect a minimum number of volunteers. And early evaluations are typically conducted in the least vulnerable subjects, often healthy adults, so that if the reactions occur, their severity will be minimized. Successive evaluations of a particular vaccine are then conducted in progressively larger numbers of volunteers, and, as confidence in the safety-profile of the vaccine increases, in subjects in the ultimate target group, including persons who are more vulnerable (e.g., infants). With these successive studies, the complete ensemble of information about the vaccine's safety, immunogenicity, protectivity, and sometimes additional characteristics (e.g., transmissibility) is accrued. Moreover, the large number of subjects ultimately studied ensures estimation of these features in a statistically precise fashion, and, for side-effects, in a way that enables detection of relatively rare events.

Clinical trials for licensing a new vaccine candidate are generally planned in three phases. In the Code of Federal Regulations (USA), the phases of clinical trials are described by using Arabic numerals (Phase 1, Phase 2, Phase 3), while in World Health Organization publications, Roman numerals are generally used (Phase I, Phase II, Phase III). In this paper, we use Roman numerals to describe the phases of clinical trials.

Phase I trials

Phase I trials of experimental vaccines are the first human studies to be conducted after preclinical studies have demonstrated suitable safety, potency, immunogenicity, and, when possible, protectivity. The primary purpose of a Phase I trial is to rule out the possibility of frequent vaccine side-effects. Additional goals include preliminary assessment of vaccine immunogenicity, determination of an appropriate dose and regimen, and, for live vaccines, measurement of vaccine shedding. Phase I trials are typically small, often on the order of 10–50 subjects, usually enroll healthy adults, and may be done with preliminary formulations of the vaccine. Depending on whether there are concerns about potential severe side-effects and the need for biological containment of excreted vaccines, such studies may be done on an inpatient or an outpatient basis. For example, a genetically attenuated, live oral vaccine candidate might well be tested initially on an inpatient basis with containment if there is concern about transmission of fecally excreted vaccine organisms, or about the genetic stability of the candidate during the course of fecal shedding [9]. Often, Phase I studies are designed in an uncontrolled fashion.

Phase II trials

For vaccine candidates that are found to yield promising findings in Phase I trials, Phase II trials may be undertaken. The primary goals of Phase II trials are to evaluate vaccine safety and immunogenicity in larger numbers of subjects, and ultimately in the target population for whom the vaccine is intended. For live vaccines, Phase II trials may also be designed to evaluate vaccine shedding and transmissibility. Phase II trials may also further evaluate different vaccine doses and regimens.

A frequently used strategy for Phase II trials of vaccine candidates intended for infants is to initiate the studies in an older age group, often adults, and to conduct successive studies in progressively younger age groups, with the transition to each younger age group contingent on satisfactory results from the study of the previous age group. In contrast to Phase I trials, which are often done with preliminary formulations, Phase II trials typically evaluate the final formulation of the vaccine, since data from studies of preliminary formulations are not usually eligible for consideration by regulatory authorities in their deliberations about vaccine licensure. Phase II trials are typically larger than Phase I trials, sometimes enrolling 200 or more subjects. By the time that a vaccine candidate has reached Phase II, concerns that would mandate testing it on an inpatient basis have typically been resolved, and outpatient studies are the norm. In contrast to Phase I trials, Phase II trials are conventionally designed as randomized, controlled trials, and control groups typically receive a placebo or an active agent to permit blinding.

For live vaccine candidates, there may be a concern about unintended transmission of the vaccine strain from vaccinees to non-vaccinees with whom they are in contact. Special Phase II trials are sometimes conducted to assess the transmissibility of such vaccine candidates. For example, a Phase II study of the live oral cholera vaccine, CVD03-HgR, in Jakarta, Indonesia, randomly allocated pairs of sibling children within households to either vaccine or placebo, and judged transmissibility of the vaccine candidate by the rate of fecal excretion of the vaccine strain and seroconversion to the vaccine in placebo recipients [10].

Phase II trials may also be done to address environmental concerns about genetically engineered, live vaccine candidates. For CVD03-HgR, for example, open sewers outside the Jakarta homes of children who had received the vaccine were sampled to evaluate whether there was any detectable persistence of the vaccine strain during the 36–40 hours after vaccination [10].

A recent trend in vaccine evaluation is to use Phase II trials to obtain initial data on the level of vaccine protection against targeted, naturally occurring infections. What distinguishes these Phase II trials from conventional Phase III trials (*vide infra*) is that they are smaller in size (although usually larger than Phase II trials geared only to the assessment of safety

and immunogenicity) and less able to evaluate vaccine protection with suitable statistical precision than well-designed Phase III trials. Also in contrast with Phase III trials, such Phase II trials may be used to evaluate vaccine prototypes that will be later modified or augmented into final vaccines for licensure (e.g., a vaccine against a single serotype, when a multi-serotype vaccine will be required for the final vaccine), and may attempt to obtain estimates of vaccine protection based on prevention of a surrogate endpoint (e.g., the use of HIV viral load as a surrogate for the rapidity of HIV disease progression).

For certain vaccines, studies are done in volunteers to evaluate the clinical protection against an intentional challenge with the target pathogen. Such studies are sometimes termed Phase IIb trials. In these studies volunteers are typically allocated at random to receive the vaccine or a comparison agent, usually a placebo, and are then challenged at a defined interval after vaccination with an inoculum of the pathogen predicted to cause the target disease in nearly 100% of the control group. The comparative attack rate of the target disease in vaccinees *versus* controls provides an estimate of the conventional measure of vaccine protective efficacy (PE) = (1 minus the relative risk of the disease in vaccinees *versus* controls) × 100%. Estimates of vaccine protection in Phase IIb studies, which are typically small, often conducted in ca. 20–30 subjects, may be helpful in triaging vaccines that are deserving of study in larger and more expensive Phase III trials [11, 12].

In addition to providing estimates of vaccine protection, Phase IIb trials can serve to provide data on vaccine safety, vaccine immunogenicity, vaccine shedding and transmissibility (for live vaccines), and preliminary assessments of immunological responses that correlate with protection. Because such studies entail intentional challenge with pathogens, subjects for the studies should always be healthy adults. The intentional challenge also limits Phase IIb trials to infections for which there is no risk of severe acute complications, significant sequelae, or chronic infection if appropriate therapy is administered promptly upon recognition that the challenge has resulted in infection. Examples of pathogens for which Phase IIb studies have been successfully carried out include cholera, diarrheagenic *Escherichia coli*, *Shigella*, Rocky Mountain spotted fever, malaria, and influenza [13]. It is imperative that such studies be carried out by staff who are highly skilled in the diagnosis and treatment of the infections under study, and it is usually desirable to conduct the challenge phase of these studies under inpatient conditions.

Phase III trials

For vaccine candidates found to be suitably safe and immunogenic in Phase II trials, Phase III trials may be done to provide rigorous evidence about

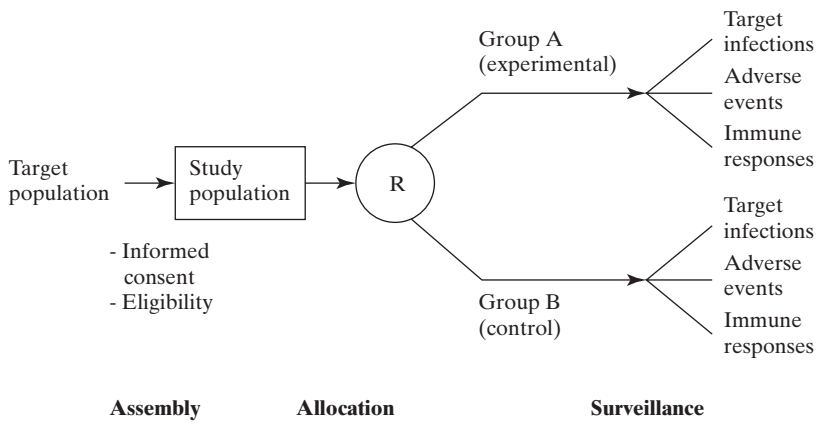

Figure 1. A simplified schematic of a Phase III vaccine trial designed as a two-group, randomized, controlled trial. In such a trial, the study population is assembled from a target population and is then randomized to receive an experimental vaccine or a control agent. The experimental and control groups are followed longitudinally and concurrently to detect the comparative occurrence of target infections, adverse events, and immune responses in the two groups to assess vaccine protection, vaccine safety, and vaccine immunogenicity, respectively.

vaccine protection against naturally occurring infections, and to provide additional data on vaccine safety in larger numbers of vaccinees. Phase III studies are designed as randomized, controlled trials with clear hypotheses, and are conducted in the target group for whom vaccine licensure is desired and in a population that normally experiences the target infection. These studies thus constitute pivotal evaluations that provide the basis for decisions about whether to license a vaccine for use in public health practice [14].

Figure 1 diagrammatically depicts the design of a Phase III trial comparing two groups. In such a trial participants are recruited from a target population and are enrolled for the study after acquisition of informed consent and ascertainment of eligibility. Prior to the trial, a formal randomization scheme is developed to allocate subjects to an experimental vaccine group or a control group, and this randomization scheme is used to allocate the compared agents to consenting subjects who are eligible for participation. To ensure blinding of investigators and subjects to the identities of the compared agents, controls may receive an inert placebo or an active vaccine. If the latter is chosen, it is typically an agent that is identical in appearance to and given with the same regimen as the experimental vaccine, but does not elicit immune responses that are known to protect against the pathogen targeted by the experimental vaccine. After randomization, subjects in the compared groups are followed concurrently with uniform surveillance procedures to detect target infections and adverse events, and the

comparative rates of these events in the two groups form the basis for the assessment of vaccine PE and safety. Similarly, participants, or a subsample of participants, are assessed immunologically at baseline and at a defined interval after dosing.

Because Phase III trials must provide statistically meaningful estimates of vaccine PE (calculated in the same way as described above for Phase IIb trials) and because the target disease outcomes to be prevented by vaccination in the trials are typically rare in occurrence, Phase III trials are often quite large, sometimes enrolling tens of thousands of subjects. In addition, because it may be necessary to measure vaccine PE over several years following vaccination in order to provide information necessary to convince regulatory and public health authorities, Phase III trials may entail maintenance and follow-up of study populations for long durations. The large size, lengthy duration, prospective conduct, and extensive quality control and quality assurance procedures needed for Phase III trials make them extremely expensive, often costing millions of dollars.

Measurement of immune responses in Phase III trials is done for two purposes. First, it is necessary to document that the vaccine tested in a Phase III trial elicited the level of immune response expected on the basis of earlier studies. Comparison of immune responses in the vaccine and control groups permits estimation of the proportion of immune responders among the experimental vaccine group that can be attributed to receipt of the vaccine. If vaccine PE proves unexpectedly low in the trial, this assessment will be crucial in helping to explain these results. Assessments to assess whether immune responses were as robust as expected usually require evaluation only of a small subsample of subjects participating in the trial. Second, it is often desirable that a trial define an immunological correlate of vaccine protection – a level of short-term immune response to vaccination that demarcates vaccinees who are protected against the target infection. Immunological correlates of protection are best defined by comparing immune responses to the vaccine in vaccinees who develop the target infection (breakthroughs) *versus* vaccinees who do not.

Definition of immunological correlates of vaccine protection is very important because such correlates permit assessments of the protection of the tested vaccine and ones suitably similar to it in small, short-term studies with immunological endpoints, without resort to full-scale, Phase III efficacy trials with clinical infection endpoints. Phase III trials usually provide the only opportunity before licensure for determining an immunological correlate of protection. Unfortunately, determination of immunological correlates within Phase III trials can be logistically demanding and costly. These problems result from four considerations: 1) it is necessary to contrast short-term immune responses in vaccinees who develop the target infection *versus* those who do not in order to determine an immunological correlate of protection; 2) at the time of vaccination and collection of specimens for immunological assessments it is unpredictable which vaccinees

will acquire the target infection ("breakthrough events"); 3) most Phase III trials have very few breakthrough events; and 4) blinding of Phase III trials prevents knowledge of who received the vaccine and who received the control agent. The implication of these considerations is that it may be necessary to obtain suitable specimens from virtually every participant in the trial to ensure that immunological assessments will be available for a large enough number of vaccine breakthroughs to enable statistically meaningful assessments. Moreover, even when arrangements are made to collect the necessary specimens from the required number of subjects, measurement of immune responses in these specimens may not necessarily yield an immunological correlate of the vaccine protection observed in the trial, sometimes for obscure reasons [15].

Other considerations for phased testing of vaccines for developing countries

When in the phased sequence a vaccine should be tested in developing countries

Experience has demonstrated that the performance of a vaccine in affluent populations in industrialized countries cannot be assumed to generalize to persons living in developing-country settings. Examples of vaccines that performed less well in developing-country than in industrialized-country populations have included oral polio vaccine, certain live oral rotavirus and cholera vaccines, and parenteral, polysaccharide-diphtheria protein conjugate *Haemophilus influenzae* type b (Hib) vaccine [16–19]. It is therefore important that new vaccines be tested in developing countries prior to their licensure in these settings.

This raises the question, for vaccine candidates developed in the industrialized world, of when in the phased sequence the candidate should be tested in the developing world. Traditionally, it has been argued, partly over concerns about using poor populations in the developing world as "human guinea pigs", that trials in the developing world should commence only when a vaccine has been fully evaluated and licensed in the industrialized world. Recent changes in thinking have challenged this view. It is now recognized that deferring trials in developing countries to this extent carries the unwanted consequence of significantly delaying the availability of vaccines to populations in developing countries. For example, polysaccharide-protein vaccines against Hib, which have been licensed for over a decade in the United States and other industrialized countries and have nearly eliminated invasive Hib disease in children in many of these settings, are used only to a limited extent today in the developing world [20]. This inequity in use of new-generation vaccines has elicited calls for parallel testing, rather than sequential testing, of new vaccines in industrialized- and developing-

world populations. Parallel-testing of new vaccines in the industrialized and developing worlds is now being pursued for both experimental HIV and rotavirus vaccines [21, 22].

Another important trend affecting the testing of vaccines in developing-country populations is the emergence of highly qualified vaccine producers in the developing world, some capable of developing innovative new vaccines. Clearly, it is appropriate that the initial groups for testing of an experimental vaccine developed by such producers be selected from the population of the country in which the vaccine is developed.

Progression of phased studies

Conventional descriptions of phased trials often portray a seamless progression of trials from Phase I to Phase II, and from Phase II to Phase III. In reality, the vast majority of vaccine candidates that are tested in Phase I studies do not progress all the way through Phase III testing, because of disappointing findings or because of a lack of sufficient resources to support the increasingly expensive studies in successive phases [2]. While unwelcome findings at any stage may lead to termination of the clinical development of a vaccine, this need not always be the case. For example, the live-attenuated, oral cholera vaccine, CVD103-HgR, was shown to be safe and highly immunogenic when given as a single dose of 5×10^8 organisms in an extensive series of studies in North American volunteers. However, when the vaccine was subsequently tested in Thai adult volunteers, it proved erratically immunogenic [23]. To address this problem, the vaccine developer raised the dose by one log, to 5×10^9 organisms, and found the vaccine to be suitably immunogenic but still safe in a variety of settings in developing countries [9]. The CVD103-HgR experience illustrates that successful vaccine development may require detours in the phased sequence of clinical trials in order to accommodate necessary adjustments in vaccine dose, schedule, or even formulation in response to findings that emerge during the course of clinical studies. However, because vaccines that appear promising in developed countries not infrequently fail to perform as well in developing countries, and because such detours can greatly increase the expense of clinical development, completion of clinical testing of vaccines for developing countries may be exceptionally challenging.

Alternative endpoints for vaccine licensure

The conventional sequence of phases usually culminates in one or more Phase III trials that are designed to provide pivotal evidence on the clinical protection conferred by the vaccine against the targeted, naturally occur-

ring, infectious disease. Sometimes, however, proof of clinical efficacy in a Phase III trial may not be necessary for licensure of a vaccine. For example, CVD103-HgR vaccine against cholera was licensed in Europe for use in travelers on the basis of Phase I-II studies, together with Phase IIb trials showing a high level of protection against experimental cholera in North American volunteers [24]. However, it is unlikely that Phase IIb evidence from industrialized countries will be taken as a basis for licensure of vaccines in developing countries, in part because of the discrepancies between vaccine performance in developed *versus* less-developed settings and in part because of the uncertainties about whether protection in Phase IIb studies predicts the performance of a vaccine in populations experiencing endemic disease.

In addition, if there is an accepted correlation between a certain immune response (e.g., a serological antibody titer) to vaccination and clinical protection, this immune response may sometimes be used as *prima facie* evidence for judging whether the vaccine is likely to be sufficiently protective to warrant licensure. For example, PRP-CRM_{197} diptheria toxin and PRP-*Neisseria meningitidis* outer membrane protein (OMP) conjugate vaccines against Hib were licensed for infants on the basis of Phase III evidence of clinical protection against invasive Hib disease. Subsequently, PRP-tetanus toxoid conjugate vaccine was licensed for infants in the United States largely on the basis of proven safety and the attainment of protective serum antibody titers to PRP, without corresponding evidence of clinical protection from a Phase III trial [25, 26]. Serological endpoints are also commonly used as a basis for licensure of combination vaccines for which the component vaccines have already been licensed and seroresponse criteria for these components have been established [27].

Some vaccines of public health importance are directed to diseases that cannot be reliably predicted in populations at risk and that cannot be ethically studied in Phase IIb trials. Examples include vaccines against some bioterrorism agents such as anthrax and plague, and vaccines against certain epidemic diseases such as SARS and Ebola. In these situations, decisions about licensure will have to depend on pre-clinical data, demonstration of safety in humans, and assessments of immune responses in humans (even if such immune responses are not known with certainty to be correlated with clinical protection of humans against the target disease).

Good clinical practice

In recent years, considerable attention has been devoted to the ethics, scientific quality, conduct, documentation, and reporting of clinical trials. Several organizations have produced guidelines for these activities, which are subsumed under the rubric of "Good Clinical Practice (GCP)". Guidelines for GCP have been published by the World Health

Organization, the United States Food and Drug Administration, and the International Conference on Harmonization (ICH), among others [13]. Table 1 presents the essential elements of GCP in guidelines issued by the International Conference on Harmonization [28].

The promulgation of GCP has had several implications for vaccine trials in developing countries. Increasingly, the world of vaccines is becoming globalized. Vaccines targeted for a global market may be tested in developing countries to provide initial licensure. For example, a live oral human rotavirus vaccine developed by a major multinational vaccine producer has recently received its initial licensure in Mexico. Moreover, vaccines produced by manufacturers in developing countries are now being used throughout the world. Indeed, the majority of all doses of measles vaccines now administered to children in the world are produced in India.

With the trend toward globalization of vaccines, increasing emphasis has been placed on conducting clinical trials in developing countries in a manner that conforms to international GCP standards. While the benefits of GCP trials are unquestionable, rigid adherence to GCP standards can be a double-edged sword. The expense of clinical trials has risen rapidly in recent years [29]. A portion of this increased expense arises from the extensive documentation and auditing requirements demanded by regulatory agencies as a component of GCP, together with the expense of proprietary products developed to ensure compliance with regulatory requirements, such as proprietary computer software for data management and electronic reporting of trials. While this increased expense can be borne by producers anticipating major markets for their vaccines in affluent markets, it constitutes a disincentive to the clinical testing of vaccines against "orphan diseases" affecting developing countries, for which lucrative markets are not foreseen.

The ethical guidelines for clinical trials in developing countries have been the subject of recent controversy. The ethical principles of respect for persons, beneficence, and justice, which underlie modern clinical trials, are well accepted [30]. Nevertheless, several issues continue to be debated, of which three are touched on here [31–33]. One issue concerns the threshold for the risk/benefit ratio for a vaccine to be tested in developing countries. For example, a live oral, quadrivalent rhesus rotavirus-reassortant vaccine was licensed and then withdrawn from the United States market by its manufacturer because of an association between vaccination and the rare occurrence of intussusception [7, 8]. Understandably, this action had a chilling effect on the testing of this vaccine in developing countries. A meeting of experts organized by the World Health Organization acknowledged the rationale for the company's withdrawal of this vaccine in the United States, where rotavirus diarrhea is a cause of morbidity but not of significant mortality. Nevertheless, there was agreement among participants in the meeting that the burden of mortality of rotavirus in developing countries provided an ethical justification for continued evaluation of this vaccine in these settings [34].

Table 1. Principles of Good Clinical Practice (GCP). From [28]

1. Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with GCP and the applicable regulatory requirement(s).
2. Before a trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial subject and society. A trial should be initiated and continued only if the anticipated benefits justify the risks.
3. The rights, safety, and well-being of the trial subjects are the most important considerations and should prevail over interests of science and society.
4. The available nonclinical and clinical information on an investigational product should be adequate to support the proposed clinical trial.
5. Clinical trials should be scientifically sound and described in a clear, detailed protocol.
6. A trial should be conducted in compliance with the protocol that has received prior Institutional Review Board (IRB)-Independent Ethics Committee (IEC) approval-favorable opinion.
7. The medical care given to and medical decisions made on behalf of subjects should always be the responsibility of a qualified physician or when appropriate, a qualified dentist.
8. Each individual involved in conducting a trial should be qualified by education, training, and experience to perform his or her respective task(s).
9. Freely given informed consent should be obtained from every subject prior to clinical trial participation.
10. All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation, and verification.
11. The confidentiality of records that could identify subjects should be protected, respecting privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).
12. Investigational products should be manufactured, handled, and stored in accordance with applicable good manufacturing practice (GMP). They should be used in accordance with the approved protocol.
13. Systems with procedures that assure the quality of every aspect of the trial should be implemented.

A second controversy over the ethics of trials in developing countries has focused on the use of placebos. As stated in the Declaration of Helsinki, "The benefits, risk, burdens, and effectiveness of a new method should be tested against the best current prophylactic, diagnostic, and therapeutic methods" [35]. There is agreement that a placebo would be inappropriate as a control agent for a trial if a locally licensed standard alternative vaccine exists; there is also general agreement that if such an alternative does not exist, it would be preferable to use for the control group an active vaccine against a different target infection, provided the active control does not cross-protect against the target pathogen and that use of the active vaccine maintains blinding in the trial [36]. However, it remains controversial whether a placebo can be ethically administered to the control groups in trials for which there is no suitable alternative active control vaccine and in which a licensed alternative vaccine against the target infection exists, but the alternative is not licensed in the country for the trial [37].

Yet a third controversy concerns what is owed to the participants at the conclusion of a vaccine trial. The Declaration of Helskinki states that: "At the conclusion of a study, every patient entered into the study should be

assured of access to the best proven prophylactic, diagnostic, and therapeutic methods identified by the study" [35]. Few would disagree that if the vaccine tested in a trial is found to be beneficial, it should be provided to all participants in the trial. However, controversy surrounds the boundaries for the group of persons entitled to receive the investigational vaccine at the conclusion of the study – only participants in the trial, all persons living in the same region, or all persons living in the same country? And for how long are these beneficiaries entitled to receive the vaccine?

Increasing expectations for demonstration of vaccine safety before licensure

Because vaccines are administered in public health practice to healthy individuals, most commonly children, there is a higher expectation of safety for vaccines than for many therapeutic agents. In the past, pre-licensure Phase III trials were often designed merely to evaluate the safety profile of a vaccine observed in previous studies. Accordingly, the focus of such trials was on evaluating events that were expected on the basis of earlier studies, that occurred during an interval immediately following vaccination, and that were seen with appreciable frequency. Indeed, a common tactic in older Phase III vaccine trials was to evaluate adverse events only in a small subsample of the total trial population, obviating the possibility of detecting rare but potentially significant side-effects. Modern regulatory agencies now usually require that surveillance for adverse events be undertaken for the total study population rather than for a subsample, at least for events that can be detected passively (e.g., among persons presenting to health care centers for treatment), and that this surveillance be maintained for the duration of the trial.

Increasing concern about vaccine safety has also led to other changes in the design of Phase III trials. Formerly, calculation of sample sizes for such trials was usually geared to enable detection of a certain level of vaccine protection against the target illness, with an acceptable level of statistical power. Detection of rare side-effects was not a typical goal of such calculations. The recent experience with the live oral, quadrivalent rhesus rotavirus reassortant vaccine, which was withdrawn from the United States market because of an association with a small but statistically significant risk of intussusception [7, 8], has led to more stringent regulatory requirements for pre-licensure trials of newer-generation, live oral rotavirus vaccines. The United States Food and Drug Administration, for example, has required that these newer- generation vaccines be tested in numbers of infants sufficiently large to be able to detect whether intussusception is a vaccine side-effect. Current trials of these newer vaccines have therefore enrolled samples of infants many times the number enrolled in pre-licensure trials of the live oral, quadrivalent rhesus rotavirus reassortant vac-

cine, resulting in greatly increased clinical development costs. Whether the experience with rotavirus vaccines portends regulatory requirements for substantially larger Phase III trials for other new-generation vaccines remains to be seen.

The experience in the United States with the live oral, quadrivalent rhesus rotavirus reassortant vaccine illustrates yet another issue confronting pre-licensure trials. Data from pre-licensure trials of this vaccine showed a suggestive but non-significant elevation of the risk of intussusception [38]. The United States Food and Drug Administration licensed the vaccine with the proviso that the occurrence of this potential side-effect be scrupulously monitored post-licensure. This was possible because of the well-developed systems for post-licensure surveillance for vaccine side-effects established in the United States [39, 40]. Unfortunately, post-licensure surveillance systems capable of detecting and evaluating rare but serious vaccine adverse reactions are absent in most developing countries.

The weaknesses of post-licensure surveillance systems in developing countries place an even greater onus upon pre-licensure trials in these settings to provide reassurance that a vaccine will not cause rare but serious side-effects. However, the large size and great expense of trials capable of detecting rare side-effects present a major dilemma, especially for vaccines against diseases that are primarily limited to the developing world, for which resources for clinical trials are scarce. Clearly, there is an urgent need to develop improved systems of post-licensure surveillance capable of detecting rare but significant vaccine adverse reactions in developing countries.

Recently work has begun to evaluate the methodological challenges and practical feasibility of establishing large population-based, dynamic computerized databases for evaluating potential vaccine side-effects in developing-country settings. Such databases link vaccination histories to severe disease outcomes in defined cohorts, and have been used successfully in industrialized countries for rapid and credible evaluations of putative vaccine adverse reactions [40]. One such database has been successfully established on a pilot scale in Vietnam [41]. Overcoming the challenges of establishing such databases in the diverse settings of the developing world remains a significant but important research agenda.

Concluding remarks

The basic phases of testing of vaccine candidates are relatively straightforward and widely accepted. The successive phases of clinical evaluation of vaccine candidates allow for acquisition of critical information about vaccine safety, immunogenicity, excretion, transmission, and protection in an incremental fashion, while minimizing the risks to subjects who volunteer to participate in these studies.

In recent years we have seen a burgeoning of vaccine candidates against diseases of developing countries, creating breathtaking possibilities for disease prevention in these settings. At the same time, this profusion of vaccine candidates for the developing world has added a layer of complexity to the seemingly straightforward phased sequence of trials.

The factors underlying this complexity are multiple, and include considerations about when in the phased testing sequence a vaccine developed in an industrialized country should begin testing in developing countries; about whether and how to pursue the clinical development of a vaccine when disappointing clinical results are observed in developing countries; about how to meet the scientific, financial, logistical, and ethical challenges of conducting clinical trials in developing countries that conform to contemporary international standards of Good Clinical Practice; and about how to ensure that a vaccine is acceptably safe before and after licensure. The manner in which these issues are addressed in the future will have a great bearing on the success of efforts to accelerate the introduction of new-generation vaccines into programs for the poor in developing countries.

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Practical aspects of phase 3 vaccine trials in developing countries

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Introduction

Setting up and successfully conducting a large vaccine trial is a major undertaking in any circumstances. Additional problems arise when a trial is done in a poor country with limited resources, for it is no longer acceptable for clinical trials to be conducted in developing countries in any less rigorous a way than in more affluent societies. In this chapter, some of the practical issues that face those wishing to undertake a large vaccine trial in a developing country that meets the highest standards of Good Clinical Practice (GCP) are discussed. Some of these trials will be true phase 3 studies, that is to say a trial of a definitive product used in the target population for which it has been developed and which will contribute to licensure. However, many of the same issues apply to vaccine trials that are not strictly phase 3 studies as they involve a product that is already licensed or one for which licensure is not being sought.

Background studies

Before any phase 3 vaccine trial is contemplated, it is essential that the background epidemiology of the infection against which the vaccine is directed is documented carefully in the proposed study area. This is especially important in the case of infections such as malaria and schistosomiasis whose prevalence and epidemiological pattern can vary widely over short distances, even from village to village [1].

It is also essential that immunogenicity and safety data are obtained in the area where the phase 3 trial is to be undertaken, or in an epidemiologically similar one, and not just in the country where the vaccine has been developed, as substantial differences in vaccine responses may occur between communities [2]. Prior exposure to the organism in question or to

related organisms, for example atypical mycobacteria in the case of a tuberculosis vaccine [3], may influence the vaccine's immunogenicity and safety.

Acquiring the background epidemiological and immunogenicity data necessary to undertake a phase 3 trial can take several years. An example of this is the programme of research on *Haemophilus influenzae* type b (Hib) infection in The Gambia (Fig. 1) which led to a successful phase 3 vaccine trial and to the introduction of this vaccine into routine use [4]. However, this process can be accelerated by the development of trial sites where a series of trials of related vaccines can be undertaken. Although epidemiological patterns can change over time, and this needs to be watched, evaluation of a second or third vaccine may progress much more rapidly than the first if the background epidemiology of the disease in question has been well documented and surveillance systems have been set in place.

Selection of the trial site

Selection of the site for a large phase 3 trial is often a complex process involving political, social and logistical as well as scientific considerations.

Scientific considerations

Although many factors play a role in deciding the site of a trial, an essential requirement is that the incidence of infection in the target age group is high enough for a trial of manageable size to be able to achieve its endpoint with sufficient statistical power. It is also important that the incidence of the infection is stable from year to year or known to vary in a predictable manner. Planning a trial in an area where the pattern of infection is highly unpredictable and subject to fluctuations from year to year is a risky strategy; a number of trials have come to grief when the incidence of infection fell below that predicted from studies carried out in previous years because of a drought or other climatic change.

Establishing the surveillance system needed to measure the impact of a vaccine in a developing country frequently requires provision of support to the routine curative and preventative health services. This may have an impact on the overall incidence of the infection that is the subject of the trial. For example, increasing the availability of an effective cure for malaria in the study area may result in a reduction in malaria transmission and a lower incidence of infection in the control group than had been predicted from initial epidemiological studies [5]. This possibility must be borne in mind when sample size calculations are being done. Although this indirect effect of a trial has the drawback that it may necessitate a larger sample size than had originally been envisaged, it has the major advantage of providing some benefit from participating in the trial to those in the control group.

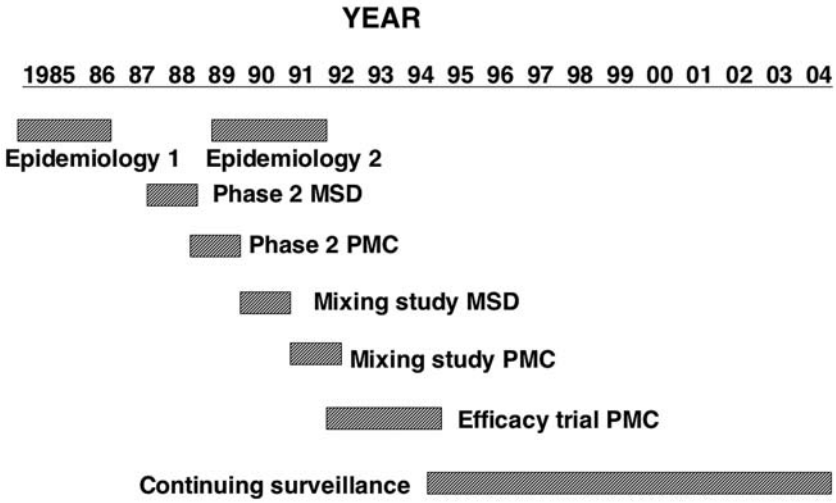

Figure 1. Time scale for a programme of Hib vaccine research in the Gambia (MSD = Merck, Sharpe & Dohme; PMC = Pasteur Merieux Connaught, now Sanofi Pasteur).

Political considerations

Large phase 3 trials are extremely demanding and difficult to accomplish in poorly-resourced countries in the best of circumstances. Thus, it is unwise to embark on a large trial in a politically unstable area, unless the objective of the trial is to investigate the impact of a particular intervention, for example measles or meningococcal vaccination, in a refugee or chronic emergency situation. Even when a politically stable site is chosen, some degree of risk remains because of the unpredictability of the political environment in many developing countries and this needs to be recognised by the sponsors. A risk-management strategy should be developed and kept up to date throughout the course of the trial.

Strong pressures may be applied by the Ministry of Health or regional health authorities to conduct a trial in a particular area for political reasons. For example, a particular health official may push for the trial to be conducted in his/her home area because of the improvements to the health services in that area that are likely to accrue and because of the job opportunities that the trial will create for those resident in his/her constituency. Provided that the area meets the scientific criteria needed to conduct the trial successfully, there are advantages in meeting requests of this kind as this is likely to guarantee a high level of political support once the trial is underway. However, problems can arise when there is a conflict between the scientific suitability of a particular site and the political imperative to conduct the trial there or when there is a change in political leadership during the course of the trial.

Political and administrative issues may influence the choice of a trial site at the local as well as at the national or regional level. Some communities operate cohesively, perhaps because of the presence of an especially able village head, whilst neighbouring ones do not. This is generally recognised although not openly discussed. Enquiries among local community workers, for example agricultural extension workers or local NGOs, may quickly identify communities that are unsuitable for inclusion in a complex trial because of poor leadership and internal dissension within the community. Failure to recognise this possibility may result in the vaccine trial becoming the basis for a struggle between different sections of the community, seriously compromising its chance of success.

Sociological considerations

Knowledge of the causes of a particular disease and the ways in which it can be prevented can vary widely from community to community over a small area because of ethnic group or religious differences. The attitudes of a community to intervention studies may be influenced markedly by the views of an individual traditional or religious leader. Collection of blood samples can be a particularly contentious issue, especially the collection of venous blood samples from small children. In some communities, the need for the collection of blood samples is well understood and accepted, whilst in others local beliefs about the consequences of blood collection, for example the view that blood is used to make medicines or sold overseas, may already be widespread or be fomented by an individual in the community who is antagonistic to the trial. Deeply entrenched views of this kind are difficult to change. It is often recognised locally that some communities are 'difficult' to work in, perhaps as a result of problems encountered in a previous research study. Such communities are best excluded from a complex trial.

Logistical considerations

Logistical factors that may influence the choice of trial site are summarised in Box 1.

Obtaining approval for the trial

A complex set of approvals is likely to be required before a large phase 3 vaccine trial can be started. This process can take many months so it is essential that it is started as soon as the trial site and study protocol have been defined. In the case of a large and complex trial, obtaining regulatory

Box 1. Practical issues that may influence the choice of trial site

1. *Access to the research centre*. Transport can account for a substantial part of the budget of a large field trial and vehicle maintenance is often a problem. Thus, whenever possible, the trial should be conducted in communities which are easily accessible by road to the centre where laboratory tests and data entry will be done.
2. *Access to an international airport*. If it is known that samples will require shipment out of the country on a regular basis, easy access to an international air-port is a bonus. This will also be appreciated by the many overseas visitors, such as trial monitors, that a large trial is likely to attract.
3. *Communications*. Mobile phones are becoming an increasingly important component of field work but areas of effective coverage are distributed patchily in many developing countries. An ability to communicate regularly with field staff and peripheral sites using mobile phones may add to the attraction of a particular site.
4. *Health care*. Participants in a trial must be provided with health care that achieves the highest standards prevailing in the country where the trial is being conducted. This may be difficult to achieve if the trial is conducted in a remote area with few health facilities. Access to health care, for example to a functional district or mission hospital, may influence the choice of study site.

and other approvals may warrant the employment of a project manager. Several types of approval are likely to be required.

1. *Approval by the regulatory authorities.* Regulatory issues are discussed in detail in the chapter by F. Verdier. If the trial is being conducted with an unlicensed product manufactured in an industrialised country, then the trial will require approval by the regulatory authorities of the country of manufacture. This will usually be accepted by the authorities of developing countries that do not have their own regulatory authority but formal approval by the Ministry of Health will usually be required. In countries that do have a regulatory body, local approval as well as approval in the country of manufacture will be needed.
2. *Ethical approval*. Ethical issues related to trials in developing countries are considered in the chapter by V.L. Tallo and H.M. Nohynek. Before a phase 3 trial can start in a developing country, approval of the relevant local ethical committee or committees is essential. In addition, if investigators from outside the country are involved, approval by their host institutions is likely to be required. Finally, if an international organisation such as WHO is participating in the trial, approval by an additional committee may be necessary. Investigators need to decide whether to submit the proposal to several committees simultaneously or to submit in sequence. The former can save time but difficulties may arise when different committees pick on different issues that each require changes to the protocol, necessitating a further round of distribution to all committees. It is generally sound practice to seek approval locally before progressing to committees outside the country where the trial will be undertaken.

3. *Research approval*. In some developing countries, all research sponsored from outside the country, whether involving interventions or not, requires approval from a central authority. This may require payment of a registration fee.

In addition to obtaining the scientific and ethical approvals required for the trial, it can be very helpful to develop formal memoranda of understanding between the partners involved in the trial, including the investigators, sponsors and local and national authorities. These should define the responsibilities of each partner in areas related directly to the conduct of the trial, but it may be helpful to include also topics such as the provision of routine clinical care for the study community during the course of the trial, training and the use of the assets acquired for the trial on its completion. Written agreements are essential as, during the course of a long trial, there may be replacement of the staff who made the initial agreements by others with different expectations.

Communication

Activities that ensure that all sections of the community understand why a trial is being undertaken, what participation will entail and the consequences of a successful outcome are always investments well made. People who need to be informed about a large phase 3 vaccine trial include central, regional and district health authorities, political and religious leaders and local health staff, community leaders, school-teachers and opinion leaders. Most importantly, efforts must be made to educate the whole community of the area covered by the trial about its objectives and not just those who will participate directly, as discussions within the community are likely to influence the willingness of families chosen to join the trial to participate. Obtaining community consent is a much more complex process than administration of information sheets to those who will be involved directly in the trial. Some of the approaches that have been used to gain community support are summarised in Box 2. Which of these activities will be most relevant for a particular trial will depend upon factors such as its size and profile but some activities, such as meetings with key opinion leaders, village meetings and the distribution of information sheets, are essential for all studies if good community participation is to be ensured.

It is generally wise to approach the dissemination process in a hierarchical manner beginning with the most senior health and political authorities before moving to village level activities.

Community meetings (Fig. 2) can be particularly helpful in ensuring that the reasons for a trial and its potential risks and benefits are properly understood, especially if the discussion can be guided by a skilled facilitator so as to provoke a debate among those attending the meeting. A trial

Box 2. Methods used to inform a community about a new vaccine trial

- Individual meetings with key political officials, religious leaders and educational and health officials in the district or region where the trial will be undertaken.
- Radio or television programmes and newspaper articles describing the background to the trial.
- Posters placed in health centres, schools and other key sites.
- Travelling singers or actors using songs or plays to describe the background to the study.
- Individual meetings with community and religious leaders at the study site.
- Presentations at schools in the study area.
- Village meetings in the study area.
- Distribution of information sheets in a comprehensible language to all households in the area where the trial will be done.

that involves a major pharmaceutical manufacturer may require, for legal reasons, lengthy information and consent forms which provide a detailed account of the procedure of the trial and its risks and benefits, but which may not be fully comprehended by the audience for which they are intended. Thus, many investigators conducting trials in developing countries now place as much emphasis on the activities listed in box 2 as a means of obtaining informed consent as on administration of the information and consent forms.

Development of the trial site

Once a trial site has been chosen, a number of activities needs to be undertaken before the trial can start. The amount of background work required will depend to some extent upon whether the site has been used previously for similar studies but it is likely to require a period of several months. Pre-trial activities that may be needed include the following:

1. *Undertaking an initial assessment of the relevant components of the local health services*. A joint assessment by members of the research team and local health services of the improvements in infrastructure, staff numbers and training that will be needed to implement the trial successfully should be carried out and agreement reached as to how the changes needed will be addressed and who will be responsible for bringing them about.
2. *Strengthening the EPI programme*. This is especially important if the trial vaccine is to be delivered through the routine EPI system. Areas likely to require support include the cold chain, transport to outreach vaccination centres and record-keeping. Although provision of extra staff for vaccination clinics and salary supplements for existing staff who undertake additional duties will improve the conduct of the trial, care is

Figure 2. Communication through a village meeting.

needed not to establish a system that cannot be sustained after the trial is over (see below).

3. *Strengthening the routine health services*. This may be required for ethical as well as logistical reasons, as discussed in the chapter by V.L. Tallo and H.M. Nohynek. If hospital admission with a particular condition comprises a major trial end-point, then in-patient services may need to be up-graded and quality control procedures introduced. In the case of trials in which out-patient diagnosis at peripheral centres is an important study end-point, some infrastructure and staff support to peripheral health centres may be needed. Strengthening of both in-patient and out-patient services may be required to ensure that any adverse events resulting from vaccination are detected and documented properly. Laboratory assays that will be used in the trial must be validated and comply with the standards of Good Laboratory Practice (GLP).
4. *Conducting or updating a census*. If the trial is to be conducted in an area which already has a demographic surveillance system in place, the quality of the data being collected should be reviewed and any deficiencies addressed. However, if no census data are available, the investigators need to consider carefully whether it is necessary to conduct a full or partial census of the study area, perhaps concentrating on the target group of special interest. Sustaining a demographic surveillance system is expensive, about $1 per person per year, and demanding of staff, so

clear evidence that this will benefit the trial is needed before this investment is made.

5. *Establishing or improving an established data management system*. Careful consideration is required before the trial starts of the type of data processing system that will be used to support the study. Expert advice may be needed to ensure that an appropriate system is set in place or that an existing system is modified to meet the needs of the trial. Factors likely to influence these decisions are: previous experience of data management at the trial site, the infrastructure available for data management, ease of communication including linkage to the internet, the availability of staff with data management experience and, if needed, the ability to recruit senior staff to work on site. Some regulatory authorities have rules about the types of database that they will accept, so they may need to be consulted. Decisions that will need to be made include:
   - Whether to employ a Contract Research Organisation (CRO) to undertake all data management or to do this "in-house" using staff employed directly by the project. However, whichever route is taken, it is important that the opportunity provided by a large trial to contribute to local capacity development should be taken.
   - Where data entry will take place. There are advantages in undertaking data entry close to the trial site so that queries can be detected soon after data collection and remedial action taken. However, improvements in communication, which allow rapid transfer of large data sets, are making this less essential than was previously the case.
   - Whether to use a paper or an electronic system for the collection of data or a combination of the two with key entry on scanned documents. Electronic systems are likely to play an increasingly important role in large trials in the future although they are still not widely accepted by regulatory bodies.

   Whatever system is put in place, it is essential that all relevant members of the trial team are involved in establishing the system, understand how to use it and appreciate the need to collect high-quality data throughout the length of the trial. Standard operating procedures (SOPs) need to define clearly who has responsibility for following up queries and who has authority to make changes on paper forms or to the data base.
6. *Monitoring*. The investigators, acting in conjunction with the trial's sponsors, need to establish systems for monitoring the trial. A quality management plan is a useful tool for the principal investigator and should be developed early. The trial's sponsors will require monitoring of data collection and management procedures so as to comply with GCP. If the trial is sponsored by a pharmaceutical company, this may be done by staff from the company. Alternatively, staff from a CRO may be hired for this purpose. In addition, a number of independent investigators have been trained in GCP by WHO and other organisations and may be available to undertake the monitor's role.

It is likely that the sponsors and the investigators will wish the trial to be guided by an independent Data and Safety Monitoring Board (DSMB). The terms of reference for this group need to be established carefully before the trial starts; they are likely to include monitoring adverse events, conducting an interim analysis of the trial results when this is appropriate and making recommendations on the extension of the trial or its premature termination. When the majority of the members of a DSMB are based outside the country where the trial is being conducted and communications are difficult, it can be helpful to have a local safety monitor based at the trial site who can act on behalf of the DSMB and who can provide immediate advice to the investigators.

Training

Training is such an important pre-trial activity that it warrants separate discussion. The extent of training required for a particular trial will be influenced by whether the trial site has been used for a major intervention study before and whether a team of trained staff can be inherited for the new study. However, even if the latter is the case, a substantial degree of training may be required.

Who needs to be trained?

Training may be required for a wide range of staff including some or all of the following:
- Senior clinical staff involved in making measurements in study individuals,
- Health services staff who will be responsible for the routine medical care of subjects in the trial when they are ill,
- EPI staff involved in vaccine administration and documentation,
- Laboratory staff,
- Field staff involved in the follow-up of study subjects,
- Data entry clerks.

There may be substantial changes in staff during the course of a long trial so repetition of courses held before the trial starts may be needed as it progresses.

Training methods

The key to a successful training programme for a large vaccine trial is the development of a detailed set of SOPs that sets out clearly who is respon-

sible for each component of the trial and exactly what they are required to do. SOPs are needed for activities such as the use of specialised equipment and transport as well as for the conduct of clinical and follow-up procedures. SOPs can be bound into an overall document that becomes the foundation for the trial and the basis for training sessions. This must be a flexible document that allows changes to established SOPs to be made and new SOPs to be developed as the trial progresses.

It is very important that staff at all levels are given an opportunity to contribute to the development of SOPs and questionnaires. This can usually be done best at workshops held before the trial starts. Workshops provide an opportunity for pilot testing of questionnaires, translation into relevant local languages and back translation. This is needed to ensure that comparable information is collected across the trial community which may include several linguistic groups. For example, finding the correct way to elicit a history of convulsions that is uniform across several language groups requires skill and may involve several iterations of the questionnaire.

Uniformity in the description of clinical findings

A trial may require detection of a clinical sign that contributes to an important trial end-point such as respiratory indrawing or pallor. When clinical observations are made by several investigators, it is important that they are consistent. Thus, an important pre-trial training activity can be clinical sessions aimed at reducing inter-observer variability in the detection of clinical signs and the interpretation of radiographs. An important recent development that has facilitated the conduct of trials that employ radiological signs of pneumonia as a trial end-point has been the generation of a consensus between experts on how to do this and production of a set of training films that are available on a CD [6]. Refresher courses may be needed to ensure that comparability in clinical observations between observers is sustained throughout the trial.

Good clinical practice and ethics

All phase 3 vaccine trials conducted in developing countries should follow the requirements of GCP as set out in the International Conference on Harmonisation (ICH) and similar guidelines (http://www.who.int/medicines/library/par/ggcp/GCPGuidePharmatrials.pdf). This may require specific training for various groups within the study team on issues such as record keeping, data management and good laboratory practice. Training in ethics may be required for senior staff and this is now available through various residential and on-line courses.

Safety

Safety of the staff conducting a trial is now seen to be as important as the safety of those participating in the study. This may require some specific training on issues such as the collection of blood and other biological samples and the safe use of project transport such as the need to use a crash helmet or seat belt.

Conduct of the trial

The first few months of a large trial are likely to make heavy demands on the study team which lessen as staff begin to work effectively together and become familiar with the SOPs for which they are responsible. However, in the case of a trial that lasts for several years, a new set of problems emerges as the trial progresses. Maintenance of the high standards set at the beginning of the study can be a particular problem. Some of the issues that may arise during the course of the trial are discussed in this section of the chapter.

Management

Management of a large vaccine trial is a complex process covering a wide range of activities including:
- Staff management. A large trial may employ several hundred people who need support on personal issues.
- Financial management. A large trial may have several donors so that managing its finances and financial reporting systems can be complicated.
- Management of a transport fleet. This may be large and demanding of resources.
- Maintenance of equipment.
- Dealing with emergency situations resulting from natural or man-made causes.

Although the host institution may have administrative staff who work in each of these areas, the demands of a large trial may be too large for them to cope effectively with the trial in addition to their other responsibilities, and the trial may need its own administrative team. Senior scientific staff should not be required to undertake all these functions.

Logistics

As the trial progresses, vehicles are likely to have accidents, vaccine refrigerators to break down and essential laboratory and x-ray equipment to

expire under the strains of operating in an inhospitable environment. Maintaining the logistical support for a large phase 3 vaccine trial can make heavy demands on the study team. It is essential that when the initial budget for the trial is developed, provision is made for the replacement of vehicles and essential items of equipment during the course of the trial if this will take several years to complete – the life-time of a motorcycle used every day on bush roads is only two to three years. Inclusion in the initial budget of a contingency fund for unexpected equipment failures is wise if the donors will allow this.

Staff

Maintaining staff enthusiasm and morale during a long trial can be difficult for staff who may be required to collect the same kind of clinical or laboratory data over a prolonged period without any apparent outcome. This can tempt staff into taking short-cuts or even into inventing data. A number of approaches can be used to sustain staff morale in addition to those of good, general staff management. These include:

1. *Regular staff meetings*. Regular staff meetings for all categories of staff, at which problems arising during the conduct of the trial can be discussed, are essential.
2. *Refresher training courses*. Lessons learnt during workshops held at the beginning of the trial need to be re-inforced by follow-up workshops and discussion groups on topics relevant to the conduct of the trial.
3. *Staff awards*. Introduction of a competitive element in which staff are given awards based on performance during the past month can help to maintain enthusiasm among those required to collect or enter repetitive data.
4. *Changing staff roles*. It may be possible to alternate staff between different roles, for example between collection of questionnaires and data entry.
5. *Ancillary studies*. It may be possible to establish studies related to the main trial which provide an opportunity for team members to take a leading role.

However, regardless of how well staff have been looked after during the course of a long trial, as the end of the study approaches staff will seek new employment and the most able staff are likely to be the first to obtain a new position. This can make the last few months of a large trial difficult to manage. This problem is difficult to avoid unless the trial is conducted at an established site which has a core complement of trained staff on long-term contracts and where a series of trials can be planned to take place in a sequential manner.

Maintaining standards

The most important way of ensuring that standards are maintained throughout a trial is to ensure that its staff are well trained and well supported. However, regular evaluation of the performance of individual members of staff is essential to ensure the maintenance of standards. Some under-performers may benefit from additional training whilst, inevitably, some will need disciplining. Disciplinary procedures must be transparent and understood by everyone who joins the trial team.

In addition, some form of external control over standards may also be required. In the case of a study that is being undertaken to support licensure of a new product, it is likely that the pharmaceutical company that has developed the product will establish a detailed system of monitoring that involves regular visits to the site by a team of monitors. This should ensure that the quality of the record keeping and data entry is maintained throughout the trial and that it meets full GCP standards. However, some additional measures may be needed to ensure the accuracy of laboratory data. A quality assurance system should be established to ensure that the quality of laboratory observations is maintained and it may be possible to introduce other quality control procedures. For example, 10% of the x-rays collected during the course of the Gambian pneumococcal conjugate vaccine trial have been read by an independent panel and the results communicated to the two primary readers.

After the trial is over

On completion of a large and complex clinical trial, local staff are likely to move on to new studies and senior staff to take up new positions, perhaps in a different country. However, their responsibilities and those of the trial's sponsors do not end with the completion of the study. Some of the post-trial issues that must be addressed are considered in the final section of this chapter.

Communication of the results of the trial

A prime responsibility of the investigators is to ensure that the results of the trial are communicated to the community where the trial has been done, even when the results are disappointing. It is essential that the results of the study are presented first to the health authorities in the area where the trial has been done and to the trial participants, perhaps through a series of community meetings, before the results are disseminated internationally. Health officials are understandably annoyed if the first information that they receive on the outcome of an important trial conducted in

their country comes from a report on the BBC's overseas service or from a poster at an international meeting.

Maintaining improvements established during the course of the trial

As discussed earlier in the chapter, it may be necessary to improve the routine health care delivery system in order to undertake a large vaccine trial in a country with limited resources. The investigators and their sponsors have an ethical responsibility to ensure that as many of these improvements as possible are sustained on completion of the study. This may not be easy to accomplish: for example, additional staff may have been employed to support clinical or immunisation services and their sudden withdrawal can be very disruptive. However, it may be possible to persuade the Ministry of Health to maintain support for some additional staff when it has been shown that their employment greatly increases the quality of care provided, for example provision of a laboratory assistant to read malaria blood films in an out-patient clinic. When withdrawal of additional staff cannot be avoided, this should be done in a phased and orderly manner that has been agreed previously with the health authorities. New and more efficient practices, such as the way in which children are managed in a vaccination clinic, may have been introduced during the course of the trial that can be sustained subsequently without added costs. Finally, vehicles and equipment may be bequeathed to the government health services on completion of the trial but these may be of little value unless funds can be found to support their maintenance and running costs. It is important that at the beginning of a trial, a plan for hand-over and maintenance of equipment purchased for the trial is developed and agreed.

Introduction of the new intervention

The most contentious issue likely to result from a successful trial in a developing country is the question of the introduction of the intervention if it has been found to be highly effective [7]. In a wealthy country, the national health authorities will usually take up fairly rapidly a new intervention that has been shown to be highly effective and cost-effective. However, this may not be the case in a developing country where there are many conflicting priorities for a limited health budget, even when it has been shown that the new intervention is cost-effective.

It is generally accepted that the investigators and the sponsors have a responsibility to those involved directly in the trial. For example, if a new vaccine is shown to be highly efficacious, a strong case can be made for offering it to those in the control group if they are still at risk. This has the disadvantage of making it impossible to determine the duration of the pro-

tective effect conferred by the vaccine but this approach will usually be required on ethical grounds. The information sheet and consent form provided to the trial participants at the beginning of the trial should set out clearly what is planned in this respect.

A more contentious issue is whether the investigators or their sponsors have any responsibility to provide the intervention to a wider group, for example the whole community where the study was done or even the whole region or country. Pressure on the company is likely to be particularly strong if the results of the trial have helped in the registration of a product that will be used widely outside the country where the trial was done and which is highly profitable to the manufacturer. However, if the company agrees to make a donation, how big should this be and for how long should vaccine be supplied? There are no easy answers to these questions and views on what is appropriate are evolving. However, it has become clear that the time to begin to address these issues is not after the trial is over but before it has begun.

Conclusion

The international community is at last paying appropriate attention to some of the major health problems of the developing word such as HIV, tuberculosis and malaria and funds for the control of these infections are becoming available on an unprecedented scale through organisations such as the Global Fund on AIDS, Tuberculosis and Malaria and the Global Alliance for Vaccines and Immunization (GAVI). Much can be done by implementing existing interventions but new tools, including vaccines, are needed if these infections are to be finally brought under control. Thus, there is likely to be a major expansion in the number of clinical trials conducted in developing countries in the next few years. Lessons learnt about the conduct of large vaccine trials in developing countries during the past few years, some of which have been discussed in this chapter, should help to ensure that these trials are conducted ethically and efficiently.

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Vaccination programmes

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Issues to be considered for the introduction of new vaccines into national vaccination programmes

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Introduction

In 1974, the World Health Organization (WHO) recommended six vaccines for worldwide routine use within the Expanded Programme on Immunization (EPI), namely vaccines against tuberculosis (BCG), poliomyelitis (OPV), diphtheria-tetanus-pertussis (DTP) and measles[1]. In 1988, WHO recommended yellow fever (YF) vaccination of all infants in countries endemic for this disease and in 1992 hepatitis B (HepB) vaccine was added to the EPI list. In 1997, the scientific advisory group of the WHO global immunization programme endorsed use of the conjugated vaccine against *Haemophilus influenzae* type b (Hib), according to national capacities and priorities, in countries where Hib disease is recognized as a public health problem. Since 1998, the WHO policy concerning the use of novel vaccines has been published in the Weekly Epidemiological Record (http://www.who.int/wer).

Whereas the initial six vaccines cover approximately 80% of the annual global birth cohort, introduction of the vaccines against YF and HepB into the national immunization programmes (NIPs) has been relatively slow. As of August 2004, 33 of the 44 at-risk countries in Africa and Latin America have included the YF vaccine in their national programmes, but in many high-risk areas less than 50% of the young children were covered by this vaccination. Of the 214 countries and territories reporting to the WHO, about 160 had adopted infant vaccination against HepB. By 2003, the Hib vaccine had been introduced into the programmes of 88 of the 192 WHO member states, most of which belong to the industrialised world. Several other vaccines, such as the rubella, typhoid, and pneumococcal vaccines, are of documented public health value, but are clearly underused so far. It is therefore important to identify common hurdles to the introduction of new vaccines and to search for mechanisms that may facilitate world-wide acceptance of essential childhood immunization.

This chapter reviews some of the issues to be considered before appropriately licensed, new vaccines may be introduced into the NIPs. Some

examples are added to illustrate issues that are characteristic of particular vaccines. Emphasis is on introducing new vaccines into NIPs of developing countries. It is realised, however, that also in developing countries considerable vaccination activities may occur outside the national programmes. In some major cities of the developing world as much as 25–45% of all vaccinations are carried out in the private sector [2]. Immunization in the private sector may therefore have considerable impact on the local epidemiology of the target diseases.

The decision-making process

Decisions to adopt new vaccines into a NIP are commonly based on assessments of disease burden, vaccine characteristics such as protective efficacy, safety and price, cost-effectiveness analyses, professional as well as public acceptance of the vaccine, as well as on programmatic and socio-economic considerations. Around the world, there are a number of organizational models for this decision process [3]. In most countries the Ministry of Health (MOH) appoints one or more advisory committee(s) whose mandate includes evaluation of new vaccines for the national programme. To avoid possible conflicts of interest, the professional and economic integrity of members of the advisory committees should be ensured and the decision process kept transparent. Suggestions for new vaccines may originate from experts within the MOH, the medical community (usually paediatricians), pressure groups such as concerned parents, international agencies such as the WHO and UNICEF, non-governmental organizations, or from the vaccine manufacturing industry. Occasionally, major outbreaks of vaccine-preventable diseases may prompt large-scale vaccination as a consequence of broad public demand. In most cases coinciding input from several different sources are at play.

Even when recommended by the advisory committee, the introduction of new vaccines may sometimes be delayed by opposition from the ministry of finance, the anti-vaccine lobby, and occasionally by the vaccine programme managers who may be already overwhelmed with the existing programme. Although inclusion of new vaccines into the NIP is ultimately a question of political will, at least in democratic settings, politicians are strongly influenced by public perception of the magnitude and urgency of the problem.

Disease burden

National records of disease incidences before and after introduction of large-scale immunization provide pertinent information on programme performance and effectiveness of the vaccine. Unfortunately, most devel-

oping countries lack the infrastructure required for comprehensive infectious disease surveillance. On the other hand, it may not be necessary that every country undertake full-scale epidemiological surveys before introducing a new vaccine. Where the actual disease is recognized as a major public health problem, surveillance from comparable epidemiological settings, possibly combined with limited national prevalence studies, may suffice to justify the introduction of documented safe and effective vaccines. In some situations, targeted serological surveys can provide the necessary information on the burden of the target disease. For example, screening of hepatitis B surface antigen (HBsAg) in representative segments of the population permits acceptable estimates of the burden of chronic hepatitis and hepatocellular cancer in the population at large. Similarly, serological surveys of rubella susceptibility in adolescents will allow qualified predictions of the number of children likely to be born with congenital rubella syndrome in the population concerned. In other situations, laboratory or clinical parameters suitable for disease-burden assessments may be very difficult to achieve. For example, in most cases of bacterial pneumonia the exact aetiology remains unknown. In the case of Hib, indirect aetiological evidence was obtained from trials comparing the total incidence of pneumonia among Hib vaccinated groups *versus* the control groups [4]. Similar indirect approaches are likely to work with other vaccine-preventable infectious agents. WHO has prepared rapid assessment tools for surveillance of several vaccine-preventable diseases [5].

Although scientific assessments of disease burden are mandatory for rational planning, the figures obtained are not necessarily representative of the disease burden as perceived by the population at large. For example, in the 1970–90s Norway experienced a protracted outbreak of meningococcal disease due to *Neisseria meningitides* type B. In 1983 the incidence peaked at 10 (in the northernmost region almost 20) per 100000 inhabitants. Children below 5 years of age and adolescents were most commonly affected and fulminant meningococcal septicaemia with high case-fatality rates occurred frequently. In this situation, the burden of meningococcal disease as perceived by the general public greatly exceeded the perceived burden of cancer in the same age groups, although their respective incidence rates were similar (statistics from the Norwegian Institute of Public Health and the Cancer Registry of Norway).

The vaccine

According to the WHO [6], new vaccines for large-scale public health use should

1) meet WHO's quality requirements;
2) be safe and have a significant impact on the actual disease in all target populations;

3) if intended for infants or young children, be easily adapted to schedules and timing of the national childhood immunization programmes;
4) not interfere significantly with the immune response to other vaccines given simultaneously;
5) be formulated to meet common technical limitations, e.g., in terms of refrigeration and storage capacity;
6) be appropriately priced for different markets.

Modern vaccines that are licensed internationally have been manufactured according to strict quality requirements and have proven to be safe. In most countries, a national regulatory authority (NRA) provides the local "license" for all locally manufactured or imported medical products, including vaccines. Vaccines that have been procured through UNICEF have all been pre-qualified according to standardized WHO procedures [7]. In addition to the efficacy and effectiveness profiles, the expected duration of protection is an essential parameter in the planning process. Also, the formulation, route and number of administrations and adaptability of the new vaccine to existing programmes are important factors to be considered as they affect public acceptance of the vaccine, the workload of the programme managers, and vaccine wastage [8]. Combination vaccines have the great advantage of providing several antigens in a single dose. This aspect becomes increasingly important with the growing number of vaccines that are offered in the NIPs. In addition to decreasing discomfort for children and parents and reducing the workload for programme officers, a reduced number of injections results in less needles and syringes and thereby reduced risk of transmitting blood-borne pathogens [9].

Vaccine efficacy and effectiveness

Vaccine efficacy is a measure of the protection obtained under the relatively ideal conditions of a clinical trial, whereas effectiveness measures the level of protection achieved in the real setting of a large-scale immunization programme. Effectiveness is negatively influenced by factors such as suboptimal transportation and storage, inadequate administration of the vaccine and by possible vaccine-induced replacement of preventable serotypes of the actual pathogen. In some cases, such as with the Hib vaccine, effectiveness may be positively influenced by herd immunity. The measured effectiveness is normally lower than the corresponding efficacy, but in well-functioning immunization programmes this difference may be relatively small. When optimally applied on their respective target groups, the best vaccines will reach protection rates of at least 90%.

No vaccines will be adopted into a national programme without evidence of high disease-preventing efficacy in the target population. Ideally, this type of information is obtained nationally through well-conducted clin-

ical trials and in many countries a favourable outcome of national vaccine trials may be a prerequisite for adoption of new vaccines. However, where resources are limited or in emergency situations the decision to adopt a new vaccine may be based on experiences gained in comparable settings, for example in neighbouring countries. Naturally, trial results should always be interpreted cautiously, particularly when no comparable studies are available to confirm the findings. Different trials may arrive at dissimilar figures for protective efficacy simply because the period of observation or the outcome criteria are defined differently. Furthermore, differences in the definition of clinical disease or disease-specific mortality or in the inclusion and exclusion criteria may strongly influence the outcome of clinical studies. The problems of misclassification may be avoided using all-cause mortality rather than pathogen-specific mortality as the endpoint [10]. In trials applying serological correlates of protection, the diagnostic method and choice of cut-off values between positive and negative results are critical.

Despite comparable study design, there are many examples of vaccines that have worked well in one epidemiological setting, but less well in others. This may reflect ethnic differences in the study populations or differences in terms of average age, nutritional status or co-infections. Also local differences between prevailing microbial strains may play a role in some situations. For example, the 7-valent pneumococcal conjugate vaccine is based on the serotypes that most commonly cause invasive pneumococcal disease in western countries. This vaccine may provide no or limited protection against some important serotypes (e.g., types 1, 3, 5, 7) found in Asia and other parts of the world. In western countries the most feared manifestation of Hib is meningitis in young children, whereas in most developing countries its aetiological role in childhood pneumonia may be far more important. Obviously, the excellent results of Hib vaccination against meningitis in western countries do not necessarily imply similar success rates against pulmonary manifestations of the pathogen in other parts of the world.

Vaccine safety

Although safety issues are given top priority throughout the vaccine development process, no vaccine is absolutely free from adverse effects. To disclose possible rare complications, proper follow-up through post-licensure studies is mandatory. Where a dangerous, vaccine-preventable disease is prevalent, most people consider active immunization as an important life insurance. However, when the disease is under control, childhood immunization may appear less important and remote risks associated with vaccination rather than frequent complications of natural infection are emphasised. For example, in recent years fear of measles and oral polio vaccines rather than of measles and poliomyelitis has been reported by western media, although the risk of developing encephalitis or paralysis following

immunization is about 1000-fold less than the corresponding risks accompanying the target diseases [11]. Also, in the 1970s unsubstantiated fear of serious adverse events caused by whole-cell pertussis vaccine (wP) contributed to falling vaccination coverage and resurgence of the disease in countries such as Sweden, England and Wales, and Japan [12]. Unfortunately, there are several examples of reduced compliance with immunization following scientifically poorly documented allegations of causal links between vaccination and serious adverse effects. Such examples include vaccination against measles, mumps and rubella (MMR) allegedly causing autism and inflammatory bowel disease and vaccination against hepatitis B allegedly causing multiple sclerosis. Also the inclusion of thiomersal and aluminium in some vaccines prompted major public concern, and resulted in reduced coverage of important vaccines in some countries. Although none of these allegations were supported by independent and thorough scientific analyses [13–15] public trust in immunization has suffered significantly, and it may take a long time to rebuild confidence.

In order to be pro-active, all countries should have access to a standing committee of independent, highly qualified experts who could look into cases of possible vaccine-related adverse events as soon as such concerns are brought up. Intra-regional collaboration in this area should be encouraged. It must be realised, however, that severe events due to programme failures are much more common than severe events caused by inherent properties of the vaccine, in particular where the programme infrastructure is poor [16]. Where a causal link to the vaccine is proven or likely and where no personal or programmatic failure is identified, mechanisms should be at hand to compensate the victims from public sources.

Vaccine price

When supplied by UNICEF, the 2004 price of the traditional EPI vaccines required for fully vaccinating an infant is approximately US$ 0.8. Adding HepB vaccine increases vaccine costs to US$ 1.6, and when also Hib vaccine is added the vaccine price increases to approximately US$ 10 per infant [5]. In NIPs personnel costs, supervision and management have traditionally accounted for the largest expenses and the vaccine itself for only about 5% of all costs [17]. Where the more expensive HepB and Hib vaccines are added to the programmes, the price of vaccines increases to 40% of the total costs of immunization and becomes the single most expensive component [18]. Although current regulatory requirements guarantee safe and efficacious products, the required manufacturing process has become very expensive. The current price per dose of the sophisticated 7-valent pneumococcal conjugate vaccine is 40–50 US dollars. Such vaccines are obviously not affordable for governments of most developing countries. The limited use of HepB vaccine in many endemic countries shows that it

is economics, not the epidemiology, which dictates introduction of vaccines into national programmes [19].

Developing countries pay more than 80% of the total costs of their NIPs. Purchase of the EPI vaccines is commonly negotiated through UNICEF or other international organizations and mostly acquired from WHO-authorized manufacturers in developing countries. Also, WHO and UNICEF in collaboration with the pharmaceutical industry have launched a tiered pricing system whereby essential vaccines may be purchased at prices that are dependent on the gross national product (GNP). Countries with the lowest income *per capita* may receive up to 75–90% of the purchase costs through international funding. In recent years, several organizations, including the Global Alliance for Vaccines and Immunization (GAVI), sponsor the provision of vaccines for developing countries [20]. The support is for a limited period of time and may be gradually reduced. A well-functioning national programme is a prerequisite for international funding. It has been suggested that the minimum national contribution towards vaccine procurement should be 0.01% of the GNP. Where this percentage is insufficient to purchase the essential EPI vaccines, global funds should meet the shortfall [21]. It is essential that novel mechanisms of pricing and purchasing are fully utilized in order to speed up the introduction of new vaccines in the developing world.

Cost-effectiveness

Several methods are used to estimate whether investing in vaccines is worthwhile. Regardless of method, all estimates show that in developing as well as in developed countries, all EPI vaccines are highly cost-effective (cost per year of life gained is below national *per capita* GNP), and mostly cost-saving (ratio of benefits divided by costs > 1.0). Also the recently introduced and technically more sophisticated vaccines are cost-effective in spite of their high prices [18]. Calculations of the cost-effectiveness of new vaccines are increasingly used as a tool to measure their value in economic terms and to establish priorities between vaccines [22]. Cost analyses should have both a short-term and long-term perspective, and include direct as well as indirect costs. Direct costs include the price of the vaccine, personnel costs, transportation and storage as well as the costs of possible adverse events following vaccination, whereas indirect costs may include loss of working hours when extra visits to vaccination centres are required [23]. A prediction of the *additional* costs associated with the inclusion of a new vaccine into a NIP may be obtained through consensus cost-estimates of each component of the existing vaccination programme, and extrapolation of the extra resources needed for the new vaccine component [24].

Similarly, both direct and indirect costs must be considered when calculating the economic impact of the target disease, such as hospital costs, costs

caused by possible sequelae of the natural infection and, when applicable, wardens' absence from work during the disease. The savings achieved through vaccination include not only reduced societal costs due to control or even elimination of the disease, but also reduced private spending on, for example, treatment. When possible, the cost-effectiveness of vaccination should be compared with the cost-effectiveness of other medical interventions, such as medication. This may help overcome the perception that vaccines must be cheap in order to be highly cost-effective, in particular as vaccines protect not only individuals and groups, but society at large [9].

Standardised tools of assessment facilitate comparison between different vaccines and allow a rational choice between different programme strategies. Mathematical modelling is increasingly used to compare the effects of different vaccination policies. The WHO has developed methodology and software to estimate programme costs [5]. Cost-effectiveness of immunization is often expressed in terms of DALYs, i.e., the cost of a disability-adjusted life-year gained through the intervention. For example, the cost per DALY gained by immunization in low-income countries was 12-17 US dollars. This cost should be compared to 200-350 US dollars per DALY saved through limited therapeutic care [25].

In spite of their usefulness, it should be remembered that cost-effectiveness analyses represent approximations only, the quality of which depends upon the correctness of the underlying data. Even with increasing quality of such data, fear, grief and physical suffering remain aspects of the disease burden that are hard to compare and to translate into economic terms.

Professional and popular acceptance

It is important that paediatricians, infectious disease specialists, vaccine programme managers and other concerned experts are well informed about an extension of the NIP before a large-scale information campaign is launched for the general public. In many countries official recommendations given by medical associations on the use of vaccines carry considerable weight and have become crucial elements of vaccine advocacy. The political impact of such expert recommendations increases considerably when adopted by non-professional pressure groups, such as non-governmental organizations or groups representing victims of vaccine-preventable diseases.

Social mobilization and positive publicity through mass media are key elements of successful introduction of new vaccines into the NIP also in developing countries. Considerable efforts should be made in advance to provide concise and balanced information to the general public. Adapting this information to local conditions and perceptions is decisive for national acceptance. In addition to presenting the facts concerning the target disease and data on vaccine efficacy and adverse events, one should emphasise the

right of all children to be protected against vaccine-preventable diseases. Also, the importance of achieving high immunization coverage should be underlined. It is unwise to omit mentioning possible allegations concerning adverse effects, but independent scientific reasons for rejecting such claims must also be presented and explained carefully.

When science is played against emotion, science is likely to lose. The "anti-vaccine movement" is hardly an organization in the strict sense of the word, but rather a number of groups that for a variety of reasons argue against immunization, particularly when executed in early childhood. Some of these groups perceive immunization as "un-natural" as opposed to immunity following "natural" infection. Others advocate individual rights over the welfare of the community. Physicians recommending vaccination are sometimes perceived as promoting mainly the interests of the vaccine-manufacturing industry. Adverse effects of vaccines, alleged or real, may be used as arguments against the vaccination recommended by the medical establishment. Interestingly, the small risk of adverse events associated with vaccines appears less acceptable to the general public than the considerably higher risk associated with use of most therapeutic drugs. It is rather peculiar that the anti-vaccine movement seems to recruit most followers in countries where large-scale immunization programmes have been most successful. Unfortunately, anti-immunization attitudes are sometimes conveyed to worried parents by poorly informed members of the medical profession.

Psychologically, the concept of the single, courageous dissident fighting the medical establishment, or the desperate victim of adverse effects standing up against the powerful pharmaceutical industry has a natural public appeal. Therefore, both dissident and victim are likely to be presented very favourably by those mass media that have no ambition to provide documented facts. The immensely beneficial impact of immunization is easily lost from the headlines.

Programmatic issues

In spite of the overall success of the EPI programme, coverage is still low (40–50%) in many developing countries and in some areas vaccination coverage seems to be falling. Strong vaccination programmes with highly motivated and appropriately supported staff is a prerequisite for successful extension of the immunization services [26]. Before adding new vaccines to the NIP, there is a need to improve immunization services in communities where coverage of existing vaccines is low [27]. As a rough rule of thumb, no new vaccine should be included before coverage for the basic EPI vaccines has reached a sustainable level of at least 80%. Weak programmes are more likely to collapse than to gain strength when adding new vaccines, however important they may be. Besides, immunization against some dis-

eases, for example rubella, may cause more harm than benefit when coverage is too low (see below).

The addition of a new vaccine to an existing NIP represents an extra burden on the programme managers, distribution lines and storage capacity. Hence, as part of the planning process the programme managers' current workload, motivation and training, as well as the financial sustainability and technical capacity of the programme, should be carefully assessed. In populations where the private sector is responsible for a considerable proportion of the total vaccination coverage, as may be the case in major cities also in the developing world, the epidemiological impact of private immunization activities should be analysed.

Age-specific background data on the disease burden will disclose at which age the concerned vaccine should be offered to maximise its preventive potential. On the other hand, new vaccines should be incorporated into existing schedules in such a way that extra visits to the vaccination centre are avoided. Most new vaccines can be easily administered simultaneously with the traditional EPI vaccines. Combined vaccines result in less injections and are in principle preferable where funds are available. Regional harmonization of immunization programmes and vaccination schedules will ultimately benefit both customers and service providers. Such harmonization could also stimulate improved coverage and thereby reduce dissemination of vaccine-preventable agents from areas with low vaccination coverage.

The recent "Vaccine Introduction Guidelines" from the WHO summarize pertinent programmatic issues related to the introduction of new vaccines [5].

Socio-economic and political issues

A number of political and socio-economic factors influence the rate at which developing countries incorporate new vaccines. The most important of these factors may be the appreciation of national governments and international agencies of the value of these vaccines [28]. However, unless political as well as economic commitment is ensured for continuous long-term immunization, the addition of a new vaccine into the national programme should not normally be considered [9]. Funding for NIPs will have to compete with other important public needs. Although vaccines are the most cost-efficient tool in the field of public health, it is often easier to raise money for curative than for prophylactic purposes.

In areas where a particular disease never existed as a public health problem, for example HepB in the Nordic countries, the cost-effectiveness of HepB immunization will be low. Hence, these countries have not yet followed the WHO recommendation to immunize all infants against HepB. Similarly, in many industrialised countries it may be hard to get public

acceptance for continued vaccination against diseases that disappeared from the area many years ago, such as diphtheria, poliomyelitis and measles. It should be emphasised, however, that a high level of social acceptance of vaccines in rich countries is not only desirable in itself, but influences both the availability of vaccines in low-income countries and the industrial choices of new vaccines to be developed [29].

Vaccine-specific issues to be considered

Universal infant immunization against HepB and vaccination against YF in endemic countries are both part of existing WHO recommendations for NIPs. Several other vaccines may be of considerable public health value in countries where the respective diseases are prevalent. Examples of typical issues to be discussed when considering the inclusion of such vaccines in national programmes are summarized below.

Hepatitis B vaccine

Specific issues include: reaching the newborn within 24 hours also when delivered at home. Motivating long-term investments in health.

Infant vaccination against HepB will drastically reduce the incidence of chronic hepatitis, thereby preventing subsequent development of liver cirrhosis and hepatocellular cancer. Where perinatal transmission of HepB virus is common, for example in South-East Asia, the first of the three HepB vaccine doses should be given as soon as possible after birth, preferably within 24 hours, using monovalent vaccine. Vaccination at birth may be difficult to organize especially in areas where most deliveries take place at home. Furthermore, as the positive impact of immunization becomes apparent only decades later, it may be difficult for parents as well as health workers to fully realise the importance of this vaccine in infants [30].

Yellow fever

Specific issues include: improving YF surveillance and strengthening the infrastructure of NIPs in remote high-risk areas. Organization of immunization campaigns to avoid major outbreaks.

In African and South American countries where YF is prevalent, inclusion of the 17D vaccine into NIPs is strongly recommended. The vaccine is cost-effective, very safe and seems to induce life-long protection. Unfortunately, several high-endemic countries have not yet, or only incompletely, implemented routine childhood vaccination against YF, partly because cases tend to occur in remote areas where the infrastructure is

weak. Improved surveillance is mandatory to identify all populations at risk of infection. In high-risk areas, infant immunization against YF should aim at ≥80% coverage and in addition, mass vaccination campaigns should be organized to prevent devastating outbreaks in non-immunized segments of the population [31].

Measles vaccine

Specific issues include: achieving at least 80 % coverage in every district and subpopulation. Coping with anti-vaccine activists and allegations concerning vaccine safety.

Live, attenuated measles vaccines are very safe and effective and relatively inexpensive. Combinations of measles vaccine with rubella (MR), or rubella and mumps vaccines (MMR) are equally immunogenic, but more expensive than measles vaccine alone. The cornerstones of the WHO/UNICEF strategic plan for measles mortality reduction are improved surveillance and – to account for the extremely contagious nature of measles – global vaccination coverage of at least 80% even in remote districts. A challenge for the NIPs is to convey the scientific evidence of the safety of measles vaccine in view of the widely published allegations of vaccine-associated autism and inflammatory bowel disease [32].

Rubella vaccine

Specific issues include: achieving at least 70-80 % vaccination coverage in order to avoid an increase rather than a decrease in the burden of congenital rubella syndrome (CRS). Reaching the majority of adolescent girls and young women with rubella vaccine. Co-ordination of public and private sector immunization.

Rubella infection in early pregnancy may result in foetal lesions termed the CRS. Prevention of CRS is the rationale for large-scale vaccination against this otherwise relatively mild childhood disease. Where rubella is highly endemic, most females contract the infection before childbearing age and vaccination may not be indicated. On the other hand, rubella vaccination is recommended in countries of low or medium endemicity where a proportion of females of childbearing age remain susceptible to the infection. To ensure sufficiently high vaccination coverage and reduce the number of injections, large-scale immunization against rubella should be offered only as a combined MR (or MMR) vaccination, and only where coverage is likely to reach at least 80%. Paradoxically, partial vaccination coverage (<70%) may increase rather than decrease the CRS burden. This situation could occur in, for example, large cities with extensive private sector rubella vaccination, but no rubella vaccination as part of the NIP. It is therefore

essential that where childhood rubella vaccination is initiated, coverage should be high and sustainable. To avoid a temporal increase in the rate of CRS following initiation of childhood immunization, all adolescent girls and women of childbearing age should be offered rubella vaccination for the 12–15 years it takes to close the "susceptibility gap" [33].

Pneumococcal vaccine

Specific issues include: establishing the burden of pneumococcal disease and sub-type profiles in developing countries. Encouraging large-scale manufacturing of conjugate vaccines with optimal sub-type profiles for children in the developing world. Stimulating political will and long-term funding commitment to pneumococcal vaccination in developing countries.

Invasive pneumococcal disease in infants and young children belongs to the most important public health problems in developing countries. However, in many areas the exact burden of the disease as well as the most important pneumococcal serotypes involved are poorly defined. The traditional 23-valent polysaccharide vaccine is unreliable in children less than 2 years of age, whereas the new conjugate vaccines induce protection also in infants. The currently available 7-valent conjugate vaccine is mainly designed for western markets, whereas the 9–11-valent vaccines that are now under evaluation include more serotypes that are relevant also in developing countries. Unfortunately, the vaccine price increases with increasing number of serotypes. In developing countries pneumococcal conjugate vaccine will be available only where there is strong political will to invest in health and where the vaccine price can be brought down through long-term, large-scale delivery agreements between manufacturers, the governments concerned and international sponsors [34].

Rotavirus vaccine

Specific issues include: leadership in international public health. Funding of essential vaccines for developing countries. Co-ordinated action from the MOHs of developing countries to secure threatened, essential vaccines for their NIPs.

Rotaviruses are the most common cause of severe diarrhoeal disease in infants and young children worldwide and are an important cause of infant death in developing countries. In 1998, a tetravalent rotavirus vaccine (RRV-TV) was licensed in the United States. The vaccine provided 70% protection against all rotavirus diarrhoea and >90% protection against severe rotavirus disease in young children. Initially, the RRV-TV vaccine appeared to be practically free from serious adverse effects. However, following immunization of about one million US children, vaccine-associated

infantile intussusception was reported at a (consensus) frequency of about one per 10000 vaccinees. For this reason, the rotavirus vaccine was withdrawn from the US market. The vaccine has not been further tested since then, and is not licensed in other parts of the world. Unfortunately, the RRV-TV vaccine had undergone limited testing in developing countries prior to its licensure in the United States. A few trials in South America showed that the vaccine worked very well in some settings, less so in others. Although other rotavirus vaccines have been developed in China and recently also in Europe (licenced in Mexico), these vaccines are still unavailable in most development countries.

The underlying problems here are of medical, economic and moral nature. Should very rare, but potentially life-threatening adverse events that make the vaccine unacceptable in the western world prevent an otherwise excellent vaccine from further trials and possible use in developing countries? The answer to this question is likely to be no. Without disregarding the inherent problems, this vaccine should have been adopted by relevant international agencies and submitted to further carefully conducted trials in different parts of the developing world in close collaboration with the manufacturer and interested countries [35, 36].

A successful case: Hib vaccination in Latin America

The most important manifestations of Hib infections, namely meningitis and pneumonia, occur in children under 5 years of age, mainly in infants. The novel conjugate Hib vaccine has been shown to be very safe and in most cases highly efficient even in children below 12 months of age. Besides, through reduction of nasopharyngeal carriage, the vaccine shows considerable herd effect. In spite of limited epidemiological information from large areas of Asia and Africa, WHO considers diseases caused by Hib to be sufficiently important to warrant inclusion of this vaccine in NIPs all over the world. Hib vaccine was adopted by many industrialised countries already in the early 1990s, for example in the United States in 1990 and in Canada in 1991. However, due to mainly financial constraints, this very successful vaccine has not yet been adopted by the majority of developing countries.

The first states in Latin America to include Hib vaccine into their NIPs were Uruguay in 1994 and Chile in 1996. As a consequence, the incidence of diseases due to Hib dropped dramatically in both countries. In 1997 the Pan American Health Organization (PAHO) recommended the inclusion of Hib vaccine in the regular immunization programmes of all member states and to establish surveillance systems to monitor Hib illness and its consequences. It was also decided to use the PAHO Revolving Fund for joint purchases of vaccines. This contributed to considerable price reductions, allowing even low-income countries in the region to adopt the Hib

vaccine. By the year 2000 more than 90% of the birth cohort in the Americas had been vaccinated or had Hib included in their vaccination schedule. Furthermore, a number of the Latin American states had introduced the combined Hib-HepB vaccine, thereby adding two essential vaccines to their programmes at the same time.

The Americas were the first to eliminate polio. The governments in this region have once again shown a high level of political and financial commitment to immunization and again they have been very successful [37, 38].

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