Vaccines for Tuberculosis: Novel Concepts and Recent Progress

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INTRODUCTION

Tuberculosis (TB) is a disease rich in paradoxes. It was one of the first diseases for which the causative agent (*Mycobacterium tuberculosis*) was identified, and one of the first for which a vaccine was developed (20, 80). Antibiotics effective against *M. tuberculosis* have been widely available for a half century (72). Yet it remains one of the great killers, causing between 2 and 3 million deaths, and an estimated 8 to 10 million new infections a year (4). Most recently, the human immunodeficiency virus epidemic has caused the number of new TB cases to explode (1). Although the disease is currently only endemic in developing countries (4), epidemics in the developed world are only prevented by ceaseless monitoring for infection and treatment of potentially exposed individuals. Thus, in addition to the mortality and morbidity it causes, *M. tuberculosis* inflicts a significant economic cost on the developed and developing world alike. This situation has arisen largely because of the complicated immunology of *M. tuberculosis* infection and has led to increasing interest in developing new and better vaccines.

At the end of the 19th century, Robert Koch discovered *M. tuberculosis* as the causative agent of TB and attempted to develop a therapy using a sterile filtrate from in vitro cultures of the pathogen. This was tested in TB patients and was spectacularly unsuccessful. Not only did it fail to provide protection against the virulent organism, but the inflammatory immune responses induced in individuals with active disease were severe. Known as the Koch phenomenon, this necrotic reaction appears to be due to overproduction of several proinflammatory cytokines, but in particular of tumor necrosis factor (TNF- α) (131). Even in Koch's time, it was thought that suc-

cessful protection against TB required an inflammatory response (a supposition now known to be true) and the fact that tuberculin induced such a strong inflammatory response in the skin (the so-called delayed-type hypersensitivity response) was taken as an indication of its activity. Therefore, Koch's failure illustrates an important point for TB vaccine development: the same immune responses involved in protection against infection with *M. tuberculosis* are also involved in the pathology underlying the disease. Stimulating the first without worsening the second is the goal of current attempts to develop novel TB vaccines.

VACCINATION AND CONTROL OF TUBERCULOSIS

The first people to succeed in this task were Albert Calmette and Camille Guérin of the Pasteur Institute, who attenuated a mycobacterium related to *M. tuberculosis* (*Mycobacterium bovis* bacillus Calmette-Guérin [BCG]) by growing it in on culture medium for 13 years, monitoring its decrease in virulence in animals through this period (20). In 1921, the newly developed BCG vaccine was administered to infants in France, where it proved a resounding success, reducing mortality by approximately 90%. In the intervening decades BCG has become one of the most widely used of all vaccines, being both cheap and safe. Unfortunately, despite early success, the BCG vaccine has had limited effect against the epidemic of TB in the developing world. The reasons for this have been a matter of debate, and this uncertainty has hindered the development of new vaccines. It is only recently that we have begun to understand the limitations of BCG, which are in large part due to the relationships between *M. tuberculosis*, BCG, and other, nonpathogenic, mycobacteria. The first part of the puzzle has been a better understanding of TB and *M. tuberculosis* infection.

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Immunopathology and *M. tuberculosis* Infection

M. tuberculosis normally enters the host via the mucosal surface of the lung after inhalation of infectious droplets from an infected individual. The droplets are deposited in the alveolar spaces, where the bacteria are taken up by phagocytic cells. Ingestion of the bacteria induces a rapid inflammatory response which results in the accumulation of a variety of immune cells and, with time, the formation of a granuloma, characterized by a relatively small number of infected phagocytes surrounded by activated monocytes/macrophages and, farther out, activated lymphocytes (55). If the infection is successfully contained, the granuloma shrinks and may eventually calcify. If however, the immune response does not successfully control the bacterial replication, the granulomas increase in size and cellularity. Eventually, cell death in the hypoxic center of the granuloma leads to necrosis. If the granuloma is close to the surface of the lung, the tissue destruction caused by necrosis can breach the mucosal surface, giving rise to the prototypic symptom of TB, a persistent cough with blood in the sputum, a process referred to as cavitation. At this point the patient is highly infectious, spreading the bacteria by aerosol.

Tissue destruction in TB is not mediated by the activities of the bacteria alone; it is immunopathological in nature, and the crucial point to understand is that it is critical for the survival of both the host and the bacteria. The outer surface of *M. tuberculosis* contains a number of molecules that bind to the host's pathogen-associated molecular pattern receptors, the best characterized of which are the Toll-like receptor (TLR) family, although others certainly play a role (81). Thus, although engagement of TLRs appears to be a crucial initial step for antimycobacterial immune responses (125, 157), all clinical strains of *M. tuberculosis* express a number of molecules (both expressed on the bacteria's surface and secreted) which trigger these pathways. Interestingly, the majority of these molecules do not seem to be crucial to mycobacterial viability, and as this pathogen has a long coevolutionary history with humanity (16, 17), it suggests that their conservation serves another important function.

The simplest explanation is that *M. tuberculosis* depends on the immunopathology that promotes cavitation for spread to new hosts. A failure to stimulate inflammatory immune responses is an evolutionary dead end for the bacteria. At the same time, the same immune responses are essential for the host to control bacterial replication. This balance is clearly illustrated by the course of tuberculosis in human immunodeficiency virus-infected individuals, whose immune deficiency renders them simultaneously more susceptible to fatal bacteremia and less infectious than normal, since they cavitate less frequently than people with an intact immune response (1).

Thus, since it cannot evade the induction of cell-mediated immunity, *M. tuberculosis* has evolved to survive it. And survive it does; even if the initial infection is successfully controlled, many (perhaps all) infected individuals develop a latent infection that can persist for decades (1, 27, 69, 88, 91, 94, 114, 133).

Immunology of *M. tuberculosis* Infections

The nature of the cells responding to *M. tuberculosis* infection and how their relative contribution changes over time is an

area of obvious importance for rational vaccine development. Animal models suggest that CD4 T cells are the most important aspect of the protective response in primary infection (126). These cells remain important in chronic infection as well, as indicated by animal studies (22, 174) and the high risk of disease reactivation in patients coinfecting with human immunodeficiency virus (1). The main effector function of CD4 T cells is mediated by their cytokine production and immunity to mycobacterial infection clearly requires a Th1 response; genetic defects in either gamma interferon (IFN- γ) or interleukin-12 (IL-12) pathways are associated with extreme susceptibility to infection (30, 33, 76). However, IFN- γ production alone is no guarantee of protection against *M. tuberculosis* (2). Most, if not all, individuals respond to mycobacterial infection by producing IFN- γ , at least initially. The IFN- γ produced by both conventional (CD4 and CD8) and unconventional ($\gamma\delta$, NK, and CD-1-restricted) (82) T cells serves to activate antigen-presenting cells, boosting expression of major histocompatibility complex (MHC) and costimulatory molecules and amplifying IL-12 and IL-18 production, resulting in a positive feedback loop for IFN- γ production (Fig. 1).

The clear role for IFN- γ -dependent anti-*M. tuberculosis* effects shown by the effect of genetic defects in this feedback loop (30, 33, 76) has been shown to be due to a variety of factors. Those which have been well studied include induction of reactive nitrogen derivatives (especially nitric oxide) (5, 111, 147) which are directly toxic, alteration of phagocytic vesicle tracking/control by molecules such as LRG-47 (98), which augment lymphocyte responsiveness in response to IFN- γ after *M. tuberculosis* infection (45), and a general inhibition of the inflammatory cascade which would otherwise lead to increased production of cytokines such as tumor necrosis factor alpha (Fig. 1).

While CD4 T cells apparently contribute more to the early IFN- γ response, CD8 T cells are thought to become more important in the later phases of disease (143, 174), possibly via cytotoxic activity (156) and IFN- γ production (143, 144). The effector function of cytolytic CD8 T cells is disputed but their primary role has been suggested to be the elimination of "old" nonactive phagocytic cells resulting in release of mycobacteria that can then be taken up by newly recruited macrophages with increased antibacterial activity. This may reflect the situation in which the immune system in individuals who had controlled the acute infection attempts to respond to the changing antigenic repertoire and growth pattern of the bacteria in the chronic phase.

The model fits well with data indicating that in animal models, a deficiency in MHC class II (and therefore lack of CD4 T cells) greatly impaired the response to acute infection while a deficiency in MHC class I, was less pronounced during the acute stage but had an influence during the more chronic stages of TB (83, 130, 155). Recent studies, however, indicate that the reality is more complicated. The CD4 T-cell population includes both effector and regulatory subsets, and evidence is beginning to accumulate that the failure of immunity to primary infection may be associated with an accumulation of CD25⁺ regulatory T cells. These cells are normally associated with down-regulation of the immune response (66) and it is not yet clear whether their increase is a natural host reaction to a growing inflammatory response, or a bacterial product-driven

FIG. 1. Schematic representation of the normal immune response and the different steps where *M. tuberculosis* intervenes. The Th1 immune response to *M. tuberculosis* is central to immunity to the pathogen, and the IFN- γ and IL-12 pathway stimulated by proinflammatory factors induced by mycobacterial cell wall lipids is essential for macrophage activation, resulting in lysosome maturation and bacterial killing. *M. tuberculosis* counteracts this by manipulating the cytokine response through induction of inhibitory Th2 cytokines (IL-4 and IL-10), affecting production of IL-12 themselves, and by modulating the activity of receptor-associated transcription factors such as Stat1. The immune response is also modulated by factors that interfere with antigen-specific responses (by either blocking presentation of antigen or, conversely, promoting immune responses to nonprotective antigens). At the same time, *M. tuberculosis* deploys an impressive armory of genes to interfere with phagocytosis and lysozyme maturation and function of antigen-presenting cells.

“trick” to evade immunity. The former is suggested by the association of more severe disease manifestations in TB patients, with decreased proportions of CD25⁺ cells in both the CD4⁺ and CD8⁺ T-cell subsets (119). Thus, it appears that the outcome of infection relies on a balance between antigen-specific inflammatory and regulatory T cells in both the MHC I- and MHC II-restricted T-cell compartments.

Adding to the complexity of the immune response, it has long been known that populations of T cells restricted by unconventional presentation molecules ($\gamma\delta$ T cells and CD1-restricted T cells) are also expanded during *M. tuberculosis* infection, (170). The expanded populations of these types of cells appear to be antigen-specific, but the antigens that they respond to are nonpeptidic (for example, lipids and long-chain glycoproteins) thus increasing the number of cues that the host immune system can respond to (9, 31, 110). Like CD8⁺ T cells, the exact role of these subsets has been controversial, in part because the data from genetic knockout models have been much less impressive than those from studies in which conventional MHC molecules have been removed (78, 83, 84). Ordway and colleagues suggest that both cytokine production and cytotoxic functions may play a role (119), but that cytotoxicity by TCR⁺ $\gamma\delta$ T cells may be their primary function, consistent with earlier studies (38) whereas noncognate cytokine production in the early phases of infection by TCR- $\gamma\delta$ T cells may amplify antigen-presenting cell function (37).

Finally, CD1-restricted cells appear to primarily recognize the glycolipids abundant in mycobacterial cell walls and to distinguish these from self-derived lipids (132). Since their

receptors are far less variable than those of T cells restricted by conventional MHC I and II molecules, it has been hypothesized that they serve as a bridge between the innate and adaptive immune systems by providing the initial IFN- γ production to start the inflammatory cascade (139, 171). But just as for MHC I-restricted cells, precisely how these T cells acquire their antigen has been a hotly debated topic. It has now been suggested that they respond to products liberated by the apoptotic process (189).

Interaction of *M. tuberculosis* and the Host Immune System

If the normal immunological process, as described above, was allowed to develop undisturbed, the infection would be eliminated without the need to boost the immune system by vaccination. However, mycobacteria have evolved a very extensive battery of factors to counteract the normal immunological mechanism. The schematic (Fig. 1) provides an outline of the core pathways involved in killing of *M. tuberculosis* by phagocytic cells and known factors that interfere with this process. These interactions are discussed below but it should be stressed that a detailed accounting is beyond the scope of this review: more interactions are being defined all the time and it is quite likely that different mechanisms are involved at different stages of the infection. What is known is that *M. tuberculosis* interferes with this process at almost every stage.

Proinflammatory factors. The cell wall of *M. tuberculosis* expresses many long-chain fatty acids, the best studied of which are the mycolic acids (13, 81, 125), which strongly stimulate

host inflammatory responses, leading to granuloma formation (161), upregulation of antigen presentation and subsequent NK and T-cell responses (136). It has recently been demonstrated that the best-characterized mycolate, trehalose dimycolate, or cord factor, selectively induces a Th1 cytokine response through STAT4 signaling (115). In addition, some of these long-chain fatty acids strongly influence the survival or death by apoptosis of infected macrophages and can promote expression of a complex mixture of cytokines in addition to those that promote IFN- γ production (113, 135). The outcome of these interactions is harder to predict, since the different combinations of factors induced by stimulation of multiple TLRs is nearly infinite and can have both positive and negative outcomes, as discussed below.

Anti-inflammatory factors. Although a crucial mediator (and proxy marker) of protection, the induction of a strong IFN- γ response may not be enough by itself (2) to kill the bacteria. The mechanism remains unknown, but live bacteria or *M. tuberculosis* cell wall extracts can inhibit some of the downstream effects of IFN- γ , by reducing STAT-1 binding to transcriptional activators, providing a potential solution: IFN- γ is produced, but its effects are modulated to the bacteria's advantage (164). Production of IFN- γ also varies markedly during the infectious process. Many groups have reported that in vitro IFN- γ recall responses are reduced in patients with advanced TB (36, 40, 176) and the observation that these responses recover in most patients during therapy suggests that this unresponsive state is directly related to the disease (75, 102, 166), though again the precise mechanism has not been described.

One potential candidate for this role is the immunosuppressive cytokine IL-10. It is known that lipoarabinomannan, a major cell wall component of *M. tuberculosis*, can bind to the DC-Sign molecule, expressed on the surface of dendritic cells. DC-Sign is crucial to dendritic cell maturation, and lipoarabinomannan binding inhibits this process, decreases IL-12 production, and induces dendritic cells to secrete IL-10 (54). Consistent with this, recent studies have found that expression of IL-10 is significantly elevated in TB patients with active disease (36, 116).

Th2 cytokine-inducing factors. Recent work also suggests that *M. tuberculosis* may interfere with the balance between IFN- γ and IL-4. Naturally, given the clear importance of Th1 responses in the control of *M. tuberculosis* infection, the hypothesis that there is a change in the Th1/Th2 balance during the development of disease or protective immunity has been an attractive one. Evidence for increased IL-4 expression in TB was suggested by the finding of elevated IgE or soluble CD30 in TB patients compared to community controls in a TB-endemic region (90). This suggestion was confirmed by intracellular fluorescence-activated cell sorting analysis and reverse transcription-PCR analyses (141, 142, 173). Other factors also point to an important role for IL-4 in the development of tuberculosis.

Differences in T-cell proliferative responses to *M. tuberculosis* antigens are associated with polymorphisms in the IL-4 receptor (186), while increased production of a splice variant (IL-4 δ 2) that antagonizes IL-4 activity appears to be characteristic of individuals who are controlling TB in its latent stage (in other words, with protection after natural infection) (36).

The observation that IL-4 remains elevated in latently infected individuals, suggests that elevated levels of its antagonist, IL-4 δ 2 in healthy, latently infected individuals may be a way of coping with IL-4 production driven by the bacteria even during latent infection (48). It also implies that the balance of IFN- γ and IL-4 (and its antagonistic splice variant) is more informative with regard to disease progression than the absolute levels of any single factor by itself, a conclusion supported by studies in the cattle model of TB (185). Most interestingly, the level of IL-4 gene expression appears to correlate both with disease severity (in TB patients) (73, 74) and with risk of clinical disease in individuals who have been exposed but are still healthy (118). This result supports the hypothesis that increased IL-4 expression is not merely coincident with TB but may play a causative role. A potential mechanism has been suggested by recent studies indicating that lipids or lipoproteins from the cell wall of epidemic Beijing strains of *M. tuberculosis* may specifically induce higher levels of IL-4 and IL-13 in human cells than nonepidemic strains and that one such glycolipid is associated in animal models with inhibition of cell-mediated immunity (101, 129).

Factors that interfere with antigen-specific responses. Recent data suggest that *M. tuberculosis* attempts to avoid the consequences of the immune response induced, by expressing "decoy" molecules, which stimulate a Th1 immune response which is antigen specific, but ultimately ineffective. For example, immune responses to the 27-kDa lipoprotein of *M. tuberculosis* are characterized by strong IFN- γ secretion, but in animal models at least, these responses are not protective, and in fact, appear to promote bacterial growth (68). The highly polymorphic PE-PGRS and PPE/MPTR gene families have also been suggested as a source of antigenic variation in *M. tuberculosis* (27). Certainly, TB patients mount immune responses to PGRS proteins (e.g., Rv1759c [44]; Rv3367 [149]) and vaccination of mice with one member of the PE-PGRS family (Rv1818c) resulted in a strong but ineffective immune response against *M. tuberculosis* infection. Interestingly, vaccination with just a fragment of the same gene (lacking the highly polymorphic region) was protective (35).

A more direct intervention is the effect of the 19-kDa lipoprotein of *M. tuberculosis*, which has a negative effect on immunity to the bacterium (191), interacts with host antigen-presenting cells via TLR1 and TLR2 (162), leading to inhibition in cytokine production, antigen processing, and MHC II expression (49, 112, 120), turning what is normally regarded as a proinflammatory pathway into an anti-inflammatory one. Since TLR2 is implicated in the inflammatory response to mycobacteria (105, 172), it has been suggested that inhibition of antigen presentation and response to IFN- γ by the 19-kDa molecule may allow the bacteria to evade immune surveillance during the latent phase of infection (53, 112, 120), while still allowing the initiation of immune responses during acute infection.

Certainly, when tested as a vaccine in animal models, the 19-kDa antigen appeared to have an antiprotective effect (191) implying that some *M. tuberculosis*-derived proteins could have long-lasting negative effects on the development of protective immune responses. While there is no empirical evidence to suggest it is a problem in human disease, the presence of the 19-kDa gene—and other possibly antiprotective proteins—in

live mycobacterial vaccines remains a concern. At the very least, such proteins are of dubious value as vaccine candidates (127, 191). Taken in total, these studies indicate that *M. tuberculosis* is able to interfere with almost every stage of the host's immune response (Fig. 1) and give some insight into why it is such an effective pathogen. Countering these complex strategies in the design of novel vaccines is a daunting task.

Factors that interfere with phagocytosis and lysozyme maturation. A major component of *M. tuberculosis*'s success as a pathogen rests on its ability to survive within macrophages. The various means used to interfere with the immune system have already been briefly discussed, but in addition the pathogen is also able to disrupt the maturation of the phagosome containing it, creating an intracellular compartment that resembles an endosome rather than a lysosome, and lacking the acidic, hydrolytic environment needed to kill the bacteria. However, fusion with other vesicles and membrane remodeling and trafficking still occur, allowing *M. tuberculosis* to acquire necessary nutrients and export its own proteins (134). A wide range of genes are involved in this process. The functions of some are as yet unknown, but putative transporters, iron-scavenging molecules, and lipid-synthesizing molecules are all apparently important (122) in preventing normal phagosome maturation.

This interaction starts with the very first contact between phagocytes and *M. tuberculosis*. Bacterial surface molecules (as yet unidentified, but presumably mannose derivatives) from pathogenic (but not nonpathogenic) mycobacteria inhibit phagocytosis by some types of macrophages (159) and therefore potentially allow the pathogen to target cell types more susceptible to infection. Invasion of phagocytes which are not already activated is important for the bacteria's survival: exposure of macrophages to IFN- γ and/or tumor necrosis factor alpha before but not after infection decreases the ability of pathogenic mycobacteria to inhibit phagosome maturation and function (140, 182). This maturation is reflected in the alteration of divalent and trivalent cations (especially iron) within the vacuole, a process which appears to depend on the accumulation of ATPases and GTPases in the vacuole (79, 140). Accumulation of ATPases also seems to be essential for the decrease in pH needed to kill the bacteria (160).

The number of ways in which *M. tuberculosis* interferes with this process attests to its importance to the survival of the bacteria. Blocking GTPase accumulation appears to depend on the bacteria having access to sufficient iron (79). By holding the phagosome in a "nonmaturing state" *M. tuberculosis* prevents fusion with late endosomal/lysosomal vesicles while retaining access to early endosomal vesicles, a process dependant at least to some extent on the blocking of calmodulin-dependant Ca^{2+} flux by lipoarabinomannan, though it is not the only molecule involved (100, 177, 178). Other lipids such as trehalose dimycolate, a potent stimulator of inflammatory responses and granuloma formation, can also prevent phagosome maturation (71). Interestingly, other lipids, including mannose-capped lipoarabinomannan (52) seem to be mimics of host phosphatidylinositols. Their presence presumably interferes with the coordinated expression of phosphatidylinositols on the surface of the vacuole, by which it signifies its maturation state (23, 24). The expression inside the phagosome by *M. tuberculosis* of a eukaryotic-like serine/threonine protein kinase G that can in-

hibit phagosome-lysosome fusion and which is crucial for intracellular survival of mycobacteria suggests that mimicking host signal transduction mechanisms to modulate intracellular trafficking may be a general survival strategy for pathogenic mycobacteria (183).

VACCINE DESIGN FOR *M. TUBERCULOSIS*

The ability of *M. tuberculosis* to manipulate the host's immune response is not, of course exclusively one-sided and the host can respond effectively, as proved by the fact that most individuals infected with the bacteria do not succumb to the disease. More encouragingly, increasing the host's immune response by vaccination is also possible, as shown by the partial success of BCG vaccination. The use of the word "partial" to describe BCG's effect is based on its very variable efficacy in different trials (46, 47). In the developed world, BCG has generally shown very high and consistent efficacy, and it is often credited with helping end the TB epidemics in Europe and Japan (60). In contrast, its effect in developing countries has been far less consistent (in some studies, the vaccine had no measurable effect on TB incidence at all). The reasons for this may have more to do with vaccine trial design and the local environment than the vaccine itself (47, 188).

It now seems clear that BCG gives significant protection, but only for a limited period of time, and in addition, it is not effective in populations already sensitized to mycobacterial antigens (whether by prior BCG vaccination, exposure to environmental mycobacteria, or latent TB infection) (15, 26). This means that while BCG seems to be effective at reducing the rate of pediatric TB if given to infants (who have no prior immunity to interfere with the vaccination), that period of immunity extends at best 10 to 20 years (28, 61, 158) so that neonatal vaccination has had little effect on the rate of TB in adults (3, 15, 25).

The implications of this for vaccine design are clear: if BCG vaccination at birth can give only short-lived immunity, the options are either to replace BCG with a vaccine that gives a longer duration of protection or to design a vaccine that can be given at a later time point to boost existing immunity and provide protection in adults. Both of these approaches have advantages and disadvantages, and the vaccines now entering clinical trials include proponents of both approaches. However, one important caveat of administering the vaccine at late time points (referred to as the late booster strategy) is the fact that in highly TB-endemic regions such a vaccine will in many cases be given to already latently infected individuals. It is therefore necessary not only to develop such a vaccine as a BCG booster vaccine but also as a postexposure vaccine—an approach that brings its own set of problems and challenges. Eventually it is possible that the only practical outcome might be a hybrid approach, what can be termed a multiphase vaccine that can be administered regardless of the infectious status of the individual and with activity both in naive and already-infected individuals (Fig. 2).

Replacing BCG (Priming Vaccines)

Replacing BCG will not be an easy task; it is perhaps the most used vaccine in the world, and despite its limitations, is

FIG. 2. Schematic model of the effect of preexposure, postexposure, and multiphase vaccination. The course of infection with *M. tuberculosis* (black line) is characterized by an increase in bacterial load until the cognate immune response develops, at which point bacterial growth is reversed. In a minority of individuals (3 to 5%), the bacteria escape control and begin to expand again, resulting in clinical TB and sputum

cheap, safe, and well established. As mentioned above, neonatal vaccination with BCG seems to consistently provide significant protection against the most severe childhood manifestations of the disease, such as TB meningitis. However, most studies have reported that the efficacy of BCG lasts for no more than 10 to 20 years (28, 61, 158). In Europe, where BCG vaccination was combined with campaigns to identify and isolate adults with TB in sanatoria (or once chemotherapy was developed, to treat them), this appears to have been enough to cause a rapid decrease in incidence. However, in much of the world where TB remains endemic, the resources to track and isolate or treat new cases are lacking and BCG vaccination alone has proven unable to decrease the number of cases in adults. Thus, in hyperendemic regions such as crowded shantytowns and urban slums, the incidence is so high (in the worst cases, reaching 1,000 per 100,000) that most adults are continually exposed to infection. In such an environment, any individual without solid immunity would soon be infected. To prevent this with vaccination alone requires the stimulation of an immune response that persists for decades.

It remains unclear if this is a realistic goal. Animal models (and current research funding paradigms) are ill suited to measure vaccine efficacy over years or even decades. That means that what is most often attempted prior to human trials is to exceed the efficacy of BCG in short-term experiments, in the hope that a stronger primary immune response will also lead in turn to a longer-lasting memory response. In contrast to its record in human trials, BCG has generally provided high levels of protection in animal models (in particular in the guinea pig model) of TB (152) and although considerable progress has been made in the development of efficient subunit vaccines, there is still no reported evidence for efficacies exceeding that of BCG in naïve individuals. Therefore, by this measure, the vaccines that have showed most promise so far as a replacement for BCG are to be found among the live mycobacterial vaccines.

The first of these to enter the clinic, the rBCG30 vaccine, has recently passed phase I clinical trials (67). This vaccine was derived from a vaccine strain of BCG, which has been genetically modified to overexpress the immunodominant antigen Ag85B (67). The idea behind this vaccine was to improve BCG by expressing higher levels of an antigen that had already been shown to be protective. Furthermore, based on the observation that BCG appeared to perform well in the earliest recorded trials but poorly in later trials (25, 26), it was suggested that "modern" strains of BCG were overattenuated and had reduced immunogenicity due to a continued loss of genes from the vaccine stock (10). This may help explain the long period of protection afforded by BCG found on reanalysis of early vaccination campaigns (6).

While it could be argued that variations in efficacy could be

explained by the fact that later trials were done under different circumstances and in different populations from the earlier trials, two lines of research support the "attenuation" hypothesis. The first is the rBCG30 vaccine itself: it was reported as having a clearly improved efficacy compared to the parental vaccine strain (67). The improved efficacy of rBCG30 came as something of a surprise, since BCG possesses a functional gene for Ag85B. However, recent work has shown that overexpression of genes can alter the immune response to the antigens they encode (127), reminding us that the mere presence of a gene tells us little about its expression *in vivo* and that a gene's effects may be modulated by the presence or absence of other genes.

The second piece of supporting evidence is the observation that new vaccines based on attenuation of *Mycobacterium bovis* (the parental strain of BCG) are both more virulent and give greater protective efficacy than BCG (34). In addition, highly attenuated mutants of *M. tuberculosis*, though still protective when used as vaccines, have proven less virulent—and less protective—than BCG (137). These results are compatible with the contention that BCG has become "too attenuated." Building on the same concept, BCG into which the RD1 locus was reintroduced (allowing it to secrete the ESAT-6-CFP10 complex) was reported to be both more virulent but also more protective than the parental BCG strain (124). Interestingly, this vaccine strain (designated BCG::RD1) has recently been shown to trigger an immune response that is qualitatively more similar to that of *M. tuberculosis* than BCG. Specifically, vaccination with BCG::RD1 induced the recruitment of activated/effector T cells and dendritic cells to the lungs of subsequently infected mice far more efficiently than the parental BCG strain (99). This suggests that antigens encoded by RD1 (and lost during the attenuation of BCG) can significantly modify the type of immune response generated by a mycobacterial vaccine or that a certain level of virulence (perhaps more than that of current vaccine strains) is necessary for the optimal induction of strong immune responses by live vaccines. Efficiency aside, it is questionable if such hypervirulent vaccine strains would have the safety profile necessary for mass vaccination, especially in areas of high human immunodeficiency virus incidence (77).

The last of the BCG-based vaccines which is near clinical testing takes a different and more technically sophisticated approach. The rBCG::Δ*ureC*-*llo*⁺ vaccine is a urease-deficient BCG mutant which expresses the listeriolysin O gene from *Listeria monocytogenes*. These bacteria are unable to arrest phagosome maturation, due to the urease deficiency, and are less virulent than wild-type BCG in immunodeficient *scid* mice. This may make it safer in populations where human immunodeficiency virus infection is widespread (which, of course, also tend to be the areas where TB incidence is highest), and where

positivity. Preexposure vaccination hastens the development of this initial immune response, leading to earlier arrest of bacterial growth and preventing the infection from becoming symptomatic (A). However, in the majority of infected individuals, the initial infection may be controlled but sterile immunity is not achieved. Instead, *M. tuberculosis* establishes a latent infection which can later reactivate, causing clinical TB. Postexposure vaccination aims to strengthen immune surveillance to prevent this reactivation (B), but it cannot prevent those cases that arise during acute infection. A hypothetical multiphase vaccine will not only inhibit the infection from becoming symptomatic but will also prevent later reactivation (C). An important consequence of preventing reactivation is that if bacterial load is reduced, not only is disease prevented in the recipient, but the cycle of transmission is broken.

the BCG vaccine can cause occasional cases of disseminated BCGosis (21). In addition, the decreased pH in the maturing phagosome provides optimal conditions for the listeriolysin, which is thought to damage the phagosome membrane, allowing leakage into the cytosol and potentially increasing the amount of bacterially derived antigen available for presentation to CD8 T cells via the cytosolic scavenger pathways as previously described (29, 57).

Other groups have attempted to make new vaccines by attenuating *M. tuberculosis* instead of augmenting gene expression in BCG, reasoning that this would give the closest simulation of natural immunity occurring after *M. tuberculosis* infection. However, as noted above, *M. tuberculosis* is remarkably adept at escaping eradication by the immune response; even a cured natural *M. tuberculosis* infection does not necessarily confer life-long protection (32). It should also be noted that (in animal models) the protection induced by a cured *M. tuberculosis* infection is not always superior to that from BCG vaccination of a naïve recipient (108). Nonetheless, at least two TB vaccines based on attenuated *M. tuberculosis* are under development. One of the most important issues such a strategy needs to address is the risk that a vaccine strain based on *M. tuberculosis* could revert to a virulent form, and obviously the question of how much attenuation is sufficient (18).

The preferred approach has been to make mutations in two crucial genes to reduce the potential for reversion to a virulent form. One such vaccine, developed at the Albert Einstein Hospital in New York, a PanD⁻ Leu⁻ auxotroph of *M. tuberculosis* (137, 138), has demonstrated both safety in immunodeficient *scid* mice and protective efficacy in the highly susceptible guinea pig infection model. It is hoped that this vaccine will enter clinical trials within the next 12 months. The Δ *phoP*/R mutant, in which the *phoP* virulence factor is inactivated by the insertion of an antibiotic gene, is another vaccine that has showed significant promise in animal models (128a). The hope is that these vaccines will generate better, perhaps longer-lasting immune responses than BCG, but with the possibility of boosting that immunity at a later stage, if necessary. However, if boosting is necessary, these vaccines are unlikely to be successful in that role.

Like BCG, all of these vaccines are intended to be given to immunologically naïve recipients (essentially, that restricts their use to neonates in developing country settings). Boosting BCG-generated immunity by a second dose of BCG given at a later time point has repeatedly failed to show any beneficial effect (89). This failure is consistent with results from animal studies, which have shown that BCG (and therefore presumably any mycobacterial live vaccine) requires a period of multiplication and dissemination in the host to stimulate a protective immune response. If this is blocked by chemotherapy shortly after vaccination (51, 148), then the protective effect of vaccination is abrogated. An existing immune response (whether due to prior BCG vaccination or cross-reactive immune responses arising from exposure to other mycobacteria in the environment) appears to have the same effect, leading to the rapid clearance of BCG (11, 19, 96). This may explain both the demonstrated effect of BCG vaccination in neonates and in adult populations where skin-test-positive individuals have been excluded (25) and its failure in trials where adult (mycobacterially sensitized) individuals were included. If, as antici-

pated, new mycobacterial vaccines are unable to stimulate life-long immunity, neonatal vaccination may need to be augmented by nonmycobacterial vaccines, so-called booster vaccines.

Augmenting BCG (Late Booster Vaccines or Postexposure Vaccines)

In contrast to vaccines designed to replace BCG, late booster vaccines aim to take advantage of the widespread use of BCG by boosting immunity in young adults already primed by earlier vaccination in childhood. Approximately 3 billion people, half the world's population, have received BCG (153), and most of these live in areas where TB is endemic. Even if the protective effect of the new vaccines lasts no longer than that induced by BCG, it would still greatly reduce the number of new TB cases by reducing incidence over the peak age for TB (25 to 35 years). The "priming/boosting" dichotomy is slightly misleading. Even in areas where BCG vaccination is routine, not every person will be BCG vaccinated and not every vaccinee will have effective immunological memory. So booster vaccines will also need to be able to stimulate effective primary responses as well. However, the requirements are slightly different from those for vaccines intended to replace or compete with BCG for neonatal vaccination.

Whereas a vaccine intended to replace BCG needs to demonstrate superior efficacy to BCG to be seriously considered, booster vaccines are often no more effective than BCG at generating primary immune responses (42, 150, 187). However, they have the additional requirement that they need to be effective in sensitized as well as in naïve recipients, a test which BCG signally fails (11). Furthermore, with the high prevalence of latent TB in TB-endemic areas, the vaccines need to be designed with their potential activity in already infected individuals in mind (postexposure vaccines). This may demand the inclusion of a completely different set of gene products to target the genes that may be upregulated by the mycobacteria in response to long-term exposure to the hostile environment of the activated macrophage. This will be dealt with later in this review.

A variety of live vaccines have been developed as booster vaccines, including a recombinant adenovirus (179) and a recombinant strain of *Shigella* designed to act as a delivery system for a DNA vaccine (50). These vaccines are still in the developmental stage and it is not clear when they will be ready for clinical trials. However, one live booster vaccine is now in clinical trials (104). This is MVA-85A, a recombinant, replication-deficient vaccinia virus expressing the strongly immunogenic antigen 85A from *M. tuberculosis* (56). The vaccine has performed well in animal models (56) and now data from the first clinical trials show that it is immunogenic and apparently safe in humans (104).

Also among the first wave of new TB vaccines entering clinical trials are products based on recombinant proteins. In the past, recombinant protein vaccines have not been terribly successful at stimulating strong Th1 responses, due to the lack of adjuvants suitable for generating strong cell-mediated immunity responses in humans without generating unacceptable side effects (58, 92). For many years, the only adjuvant approved for human use was alum, which was only effective for

TABLE 1. Adjuvants for use with human subunit vaccines^a

Type	Adjuvant	Characteristics
Adjuvants suitable for use with subunit vaccines, already registered for human use	Alum (aluminum hydroxide)	The first human adjuvant, alum promotes a strong humoral response and is widely used in viral vaccines; however, it generates strongly Th2-polarized responses and is not suitable for use as a TB vaccine (14, 92)
	MF59	An oil-in-water emulsion composed of 5% squalene, 0.5% Tween 80, and 0.5% Span 85; like alum, it generates primarily humoral immunity and is used in influenza vaccines (123)
	Virosomes	Similar in structure to liposomes, virosomes are differentiated by containing viral proteins embedded in their membrane, which are delivered into host cells by membrane fusion; currently used in vaccines against viral targets such as influenza and hepatitis A, where humoral immunity is most important (70)
Adjuvants suitable for use with subunit vaccines, in or approaching clinical trials	CAP (calcium phosphate nanoparticles)	Currently in early clinical trials, CAP has been used to generate humoral responses, but the lower levels of IgE induced suggest it may not be as polarized towards the Th2 pole of the immune response as alum (62)
	LTK63	A modified and detoxified heat-labile toxin from <i>Escherichia coli</i> tested in human volunteers as an influenza vaccine; generates strongly Th1-polarized responses and is therefore being considered for vaccines against <i>M. tuberculosis</i> and HIV (121)
	Lipovac	A stable emulsion containing the cationic surfactant dimethyl dioctadecyl ammonium bromide and the synthetic mycobacterial cord factor trehalose dibehenate (64); has shown good efficacy in animal models and a good safety profile in toxicity studies; currently planned for clinical trials in a TB vaccine in 2006–2007
	AS2	An oil-in-water emulsion containing 3-deacylated monophosphoryl lipid A (a detoxified form of lipid A from <i>Salmonella enterica</i> serovar Minnesota), and a purified fraction of <i>Quillaria saponaria</i> , known as Quil A; currently in early clinical trials as a TB vaccine; a synthetic analogue of monophosphoryl lipid A called RC-529 is in clinical trials in an HIV vaccine; generates strongly Th1-polarized responses (163)
	IC31	A mixture of oligodeoxynucleotides and polycationic amino acids; generates strong Th1 responses and will enter phase I clinical trials in 2005 as part of a TB vaccine (93, 103)
	Montanide	A water-oil emulsion, two variants exist, based on mineral and non-mineral oil; tested initially as a cancer immunotherapeutic agent, Montanide has now been through a variety of clinical trials through to phase III; it generates a mixed-cell-mediated and humoral response, which may render it less attractive for a TB vaccine (7)
	ISCOM	A formulation of <i>Quillaja saponins</i> , cholesterol, phospholipids, and protein, typically self-assembling into small icosahedral cage-like particles; used initially for veterinary vaccines, immunostimulatory complexes have recently shown promise in late-phase human clinical trials for viral vaccines; their potential for <i>M. tuberculosis</i> vaccines remains unknown, as they generate a mixed humoral and cell-mediated response (109)
	OM-174	A modified and detoxified lipid A from <i>Escherichia coli</i> (diphosphate triacyl); synthetic analogues also exist; currently in early clinical trials for cancer immunotherapy and suggested for TB vaccine use; generates strongly Th1-polarized responses (107)

^a The adjuvants listed are those which are already in or about to enter clinical trials. Many of these adjuvants are also being tested as platforms; that is, the basic delivery system is being tested together with other immunomodulating agents (such as ISCOMs being used with mycobacterial lipids) or vice versa (monophosphoryl lipid A [MPL-A] derivatives are finding their way into many adjuvants, and MF59 is being augmented with oligonucleotides) and so on. These variants are in general, however, less developed with regard to clinical use.

vaccines that required a humoral response (e.g., diphtheria, tetanus, and hepatitis B vaccines). Indeed, since this adjuvant biases the immune response towards the Th2 pole (14) it has actually been shown to decrease the protection generated by vaccination against *M. tuberculosis* (92). More recently, MF59

was approved for human use, but this adjuvant has so far also been restricted to vaccines where generating humoral responses is key, such as influenza (123).

However, new adjuvants are now entering the clinic (Table 1) and several of these generate strong Th1 responses, making

them good candidates for vaccines against *M. tuberculosis*. These new vaccines owe their success in animal models to an improved understanding of the activation of the immune system in response to conserved molecules on pathogens (41, 63, 64, 93, 121, 169) being built around bacterially derived lipids, bacterial toxins, or analogues of these molecules. In some cases (monophosphoryl lipid A, for example) the receptors are known and are part of the Toll-like receptor family. In other words, we are starting to use some of the same techniques to manipulate the immune responses that *M. tuberculosis* uses which were described above. We can expect to see more vaccines using this approach in the future, since both recombinant antigens and the adjuvants can easily be synthesized on a large scale, lending themselves to vaccine production.

The first of these subunit TB vaccines to enter clinical trials is the 72f vaccine (150), jointly developed by Corixa and GSK. This vaccine is a fusion molecule comprised of two proteins, with the PPE family member Rv1196 inserted into the middle of the putative serine protease Rv0125, which is thus present as two fragments. The adjuvant used contains the saponin derivative QS21 mixed with the TLR4 ligand monophosphoryl lipid A (150). In addition to its activity for priming, this vaccine has also been demonstrated to have a BCG booster effect (12). The second recombinant protein, although developed independently by the Statens Serum Institute, is very similar in its design philosophy. It is a fusion molecule comprised of two immunodominant, secreted proteins from *M. tuberculosis* (ESAT-6 and Ag85B) and has proven highly efficacious in animal models ranging from mice to primates, more effective in fact, than the single antigens (42, 117, 184). The vaccine has also proven effective as a booster for BCG and augments its efficacy even though the ESAT-6 component of the vaccine is not present in BCG (authors' unpublished data).

The Ag85B-ESAT-6 fusion protein is slated for two clinical trials in 2005-2006. The first of these will test the vaccine in a conventional parenteral vaccination strategy, using a mixture of oligodeoxynucleotides and polycationic amino acids as the adjuvant (93). The second trial, running in parallel, will test the same antigen by the nasal route, using LTK63, a modified, heat-labile enterotoxin from *Escherichia coli* as an adjuvant (121). ESAT-6 is currently also being applied as a diagnostic tool to detect cell-mediated immunity responses that specifically signal the presence of ongoing infection (40, 87, 128, 175). New fusion molecules that do not contain ESAT-6 are therefore the subject of ongoing research and interestingly, the only antigen so far found which may be able to replace ESAT-6 is also a member of the same small gene family but is, in contrast to ESAT-6, present in BCG (39, 93, 121, 151).

In some ways these two recombinant vaccines can be viewed as *M. tuberculosis* in miniature—they contain two immunodominant antigens and use as adjuvants modified molecules derived from human pathogens that stimulate Th1 responses. This minimalist approach offers certain advantages. By choosing a limited number of carefully tested immunodominant antigens, we should generate a strong protective response, while avoiding the possibility of inducing unwanted modulation of the response or of antigen presentation. By using adjuvant molecules (slightly modified to decrease their toxicity) derived from human pathogens, we should generate exactly the type of

inflammatory response the body normally invokes to deal with a bacterial infection.

Postexposure Vaccination

All of the vaccines described above are prophylactic vaccines, intended to be given to individuals prior to *M. tuberculosis* infection, and hopefully prior to exposure to the disease. It is not clear whether they will be efficacious or even safe if given to individuals already infected with *M. tuberculosis*. For booster vaccines this is a serious issue, since in TB-endemic areas, the target population can be expected to include many latently infected subjects. What few data are available from animal studies do not suggest that the leading vaccine candidates induce a Koch phenomenon-like effect in infected recipients, but neither do they suggest these vaccines are effective against the latent phase of the disease (167; authors' unpublished work). It is likely that handling the billions who are potentially latently infected with *M. tuberculosis* will require a combined strategy, using a multiphase vaccine effective against both acute and latent infection (Fig. 2).

The acute phase of *M. tuberculosis* infection is characterized by rapid bacterial growth and the development of an immune response targeted towards bacterial antigens actively secreted in the first growth phase, such as ESAT-6 (154). The vaccines so far developed against these acute-phase antigens can reduce the severity of the initial bacteremia and disease but they do not prevent the establishment of infection (42, 117). However, as *M. tuberculosis* adapts to the hypoxic and hostile environment of host macrophages, it has been shown to undergo a dramatic change in gene transcription thought to be characteristic of latency (65, 106) and this change in gene expression may enable the pathogen to persist in the face of strong memory immune responses. This latent stage of infection is normally associated with a few bacteria surviving in a so-called "dormant state" with low or altered metabolic activity.

When grown in vitro under conditions mimicking those thought to exist in vivo, *M. tuberculosis* up-regulates overlapping, characteristic sets of genes (145, 181, 192). Increased expression of genes such as HspX (also called α -crystallin, Rv2031c, etc.) during the stationary growth phase (192) appears to be crucial for the survival of the organism (193). In agreement with these in vitro observations it has been demonstrated that the transcription of a number of genes is down-regulated while others are strongly up-regulated after the initiation of a strong immune response in infected animals (146). Together with the abundance of regulatory proteins in the *M. tuberculosis* genome (27), this indicates the importance of the pathogen's being able to adapt to different environments during infection. This regulatory flexibility may underlie its ability to shift between acute progressive disease and long-lived latent infection.

In addition to the experimental evidence described above, studies in humans are beginning to offer evidence to support the hypothesis that immune responses in healthy but latently infected individuals are targeted to different antigens than the response to an acute infection (Demissie et al., submitted; Klein et al., submitted). It is hoped that vaccinating with other antigens, such as HspX, identified by the in vitro methods described above may promote an immune response that will

help prevent reactivation of disease. There is some evidence that such an approach may indeed work. Lowrie and colleagues successfully employed an HSP65-based DNA vaccine in latently infected mice (95), but this finding has been somewhat controversial (168) and emphasizes the need for more research in this field, in particular, the need for rigorously validated animal models.

One area attracting such interest is the "resuscitation-promoting factor" or *rpf* genes. *Micrococcus luteus* expresses an *rpf* gene whose product is required to resuscitate the growth of dormant cultures of *Micrococcus luteus* and which is essential for the growth of this organism. It has recently been demonstrated that *M. tuberculosis* has at least five *rpf* homologues (43, 165), again suggestive of the importance of latency and resuscitation to the survival of *M. tuberculosis*. Given that they appear to be essential for recovery from the dormant state and are immunogenic, secreted antigens, they make tempting targets for vaccination (190).

The postexposure vaccination area is now being intensively pursued, and although a vaccine is still in the future, it offers the possibility of a multiphase vaccine that induces responses both against acute phase antigens, thus providing protection if the recipient is subsequently infected, and against latency-associated antigens, so that disease is held in remission even if the vaccine recipient is already infected.

INTO THE CLINIC: THE ROAD AHEAD

TB vaccines are only now entering the clinic, but the path for testing is already well laid out: the risk of inducing a severe reaction in already-infected individuals and the high rate of TB and human immunodeficiency virus coincidence in many areas mean that vaccines are first tested for safety and immunogenicity in immunologically naïve individuals (neither BCG vaccinated nor skin test positive) before proceeding into human immunodeficiency virus-positive and latently TB-infected individuals. Concurrent with this, booster vaccines will need to be tested in BCG-vaccinated subjects to see if existing immune responses can be boosted. The first of the new booster vaccine candidates will start this phase in 2005. Given the extensive testing that has already been done in animal models, including primates, it is unlikely that safety problems will arise. That leaves two major hurdles for new vaccines to face.

The first is the question of efficacy. The older TB vaccine trials based on BCG measured efficacy using a simple clinical endpoint: how many cases of TB in the vaccine group compared to a placebo or unvaccinated group. TB develops slowly, and even in high-incidence areas, the case rate is typically much less than 1%. This means that to obtain reliable data, efficacy studies have taken decades and involved very large numbers of participants (typically in excess of 100,000) (8). That is a costly exercise and unattractive to both vaccine developers and public health systems trying to deal with millions of cases annually. Priming vaccines such as recombinant BCG and attenuated *M. tuberculosis* must be used in immunologically naïve populations, as discussed above, so ultimately their effect on adult pulmonary TB will only be detectable after decades.

However, a quicker route to determining their efficacy in humans will be to examine the effect on TB in infants com-

pared to standard BCG vaccination. Though such a study will necessarily be large, it could be completed in only a few years, and this is the course most likely to be adopted at first. Booster vaccines have a shorter duration between vaccination and the peak years for adult TB incidence, as they are likely to be given to young adults in their teenage years. However, this still means a wait of approximately a decade before any effect can be expected to be clearly detectable. Thus, even late booster vaccines may be tested first against pediatric TB, simply to demonstrate that they are effective in humans.

Recent progress in the TB diagnostic field may turn out to be a very important tool in future clinical trials. The number of *M. tuberculosis* infections is much greater than the number of clinical cases, and recent improvements in diagnostic tools, based on measuring immune responses to *M. tuberculosis* virulence factors (such as ESAT-6, which is not expressed in BCG or most environmental mycobacteria), have shown promise at detecting subclinical and latent TB (40, 59, 85, 86, 128, 175). Recent data indicate that the magnitude of the ESAT-6 response may reflect the severity of infection (97, 180) and may predict later breakdown with disease (40) so that measuring the rate of incipient disease (and high ESAT-6 reactivity) rather than the rate of subsequent breakdown may offer the possibility of a far more rapid assessment of vaccine efficacy and the ability to use much smaller cohorts. Before it can be used, however, this approach needs to be validated, and studies to that purpose are ongoing.

The second question is what effect even an improved vaccine will have on TB incidence, given the vast reservoir of latent TB that already exists. Mathematical modeling suggests that a postexposure vaccine effective at preventing disease in latently infected individuals would cause a significant decrease in the number of new cases in the short term, but that over time a preexposure vaccine would have larger effect (194). The ideal approach would therefore be a single multiphase vaccine that was effective against both acute and latent infection, but no such vaccine currently exists. Thus, although we have come a long way in TB vaccine development in the last decade, there are still significant gaps in our knowledge that we need to fill before we can truly hope to control *M. tuberculosis* through a novel vaccination strategy.

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