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A new TB vaccine

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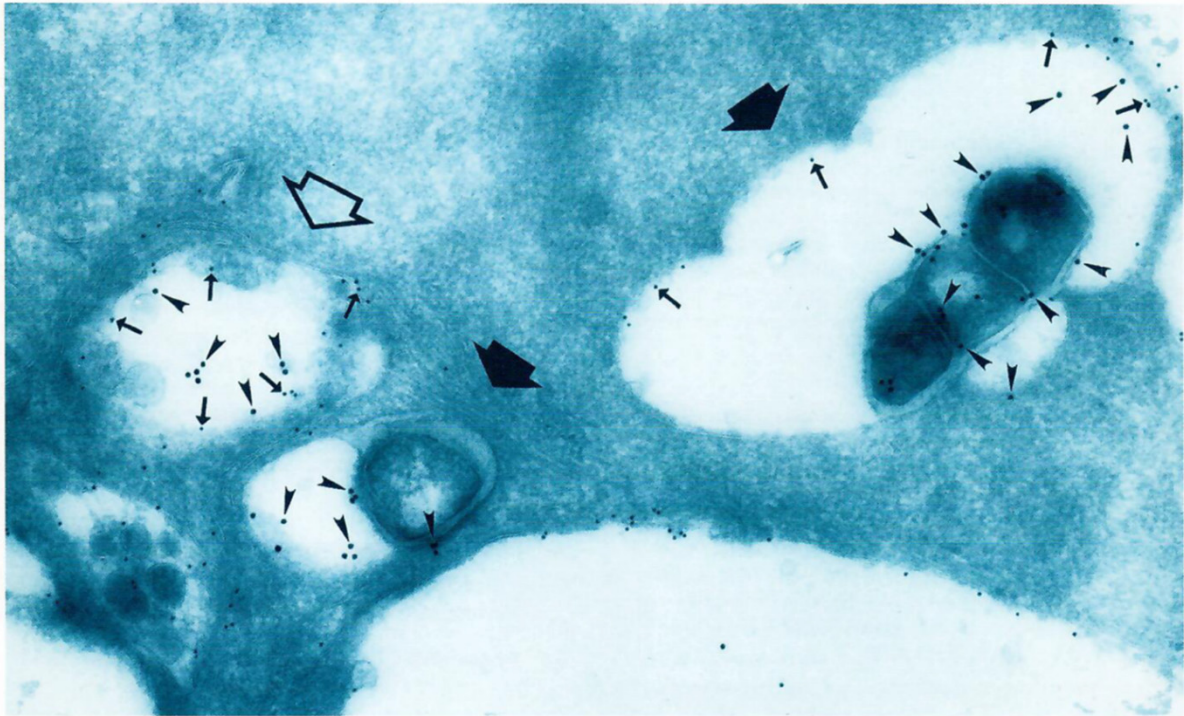
WHO's Global Programme for Vaccines and Immunization
Neonatal Immunoresponsiveness
Enhancing Cytokine Effects *In Vivo*
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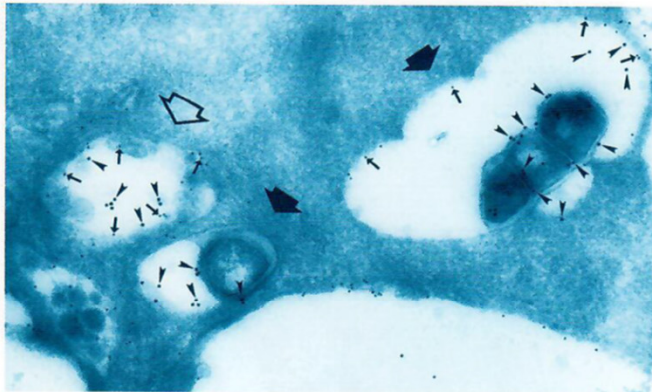
Cover
Major extracellular proteins of *M. tuberculosis* are secreted by the bacterium's phagosome in infected human macrophages: For further details, see the article by Marcus A. Horwitz on pp. 15–20.

Although *M. tuberculosis* was identified as an agent of infectious disease by Koch in 1884, it has proved difficult to produce a safe and effective vaccine against it. In this article, Marcus A. Horwitz explains the need to replace the BCG vaccine, outlines the search for new vaccine designs, and points the way to the likely future of TB vaccines.

Tuberculosis (TB) causes about 3 million deaths annually, making it the world's most lethal infectious disease caused by a single agent. A major opportunistic infection in acquired immunodeficiency syndrome (AIDS), the incidence of TB will rise dramatically over the next decade, prompting the World Health Organization to declare TB a global emergency, the first disease so designated. Worsening the crisis is the emergence of multidrug resistant tuberculosis (MDRTB), caused by strains of the primary causative agent, *Mycobacterium tuberculosis*, that are resistant to the major, and sometimes all, conventional antibiotics used to treat TB.

The Need for a New Vaccine

A safe and effective vaccine against tuberculosis offers the greatest hope for reining in this disease. Currently, the only available vaccine is BCG (Bacille Calmette-Guérin), a live attenuated strain of *Mycobacterium bovis*, a mycobacterial species that frequently infects cattle and other domesticated animals, and occasionally humans. This species is closely related (DNA >90% homologous) to *M. tuberculosis*. BCG protects against a serious form of TB, meningitis, but its efficacy against pulmonary tuberculosis is questionable. While some human trials have demonstrated protection, others have not, including the largest and most carefully conducted trial involving over 100,000 people in Chingleput, India. BCG has been called "at once the least satisfactory and yet the most widely used of all vaccines today" [1].

Aside from the issue of efficacy, BCG has several other drawbacks.

- First, it can cause serious and occasionally fatal disseminated disease in immunocompromised patients, including AIDS patients. Fortunately, such cases are uncommon.

- Second, it frequently causes the tuberculin skin test to turn positive, markedly diminishing the utility of this valuable diagnostic tool, still the gold standard of diagnostic tests for TB. BCG is not recommended for general use in the USA.

Obstacles to Vaccine Development

Why has it been so difficult to develop a TB vaccine comparable in quality to the many other vaccines in widespread use today? After all, *M. tuberculosis* was the first bacterium formally identified as an agent of infectious disease by Robert Koch in 1884. There are several reasons:

A New TB Vaccine

Marcus A. Horwitz

- First, *M. tuberculosis* is an intracellular parasite that survives and multiplies within human mononuclear phagocytes; in other words, it is an organism that uses, as its cellular host, cells of the immune system designed to fight infectious agents. In this respect, *M. tuberculosis* resembles the AIDS agent, the human immunodeficiency virus (HIV). Hence, *M. tuberculosis* is a formidable opponent.

- Second, vaccines in widespread use today target pathogens against which humoral immunity plays a dominant role in host defense. However, in the case of intracellular pathogens, including *M. tuberculosis*, cell-mediated immunity plays the dominant role in host defense – antibody has no evident role. Relatively little is known about how to formulate vaccines for the induction of cell-mediated immunity.

- Third, humans are the primary host and only reservoir of *M. tuberculosis*, and the organism is highly adapted to us. There is evidence of TB in stoneage peoples. Thus, the organism has had a long time to evolve so as to frustrate our immune defenses.

- Fourth, TB has not been perceived as a major problem for the economically privileged countries of the world which have the means and basic science infrastructure required to develop a new TB vaccine. Because of this short-sighted view, which is coming back to haunt us, both public and private support for TB research has been meager.
- Fifth, *M. tuberculosis* poses major impediments to study. It is exceptionally biohazardous, which imposes a requirement for expensive biocontainment facilities and some courage on the part of investigators. It is also exceptionally slow-growing, which places an enormous drag on the progress with which it can be studied and challenges the patience of investigators. For example, a typical experiment testing the efficacy of a vaccine in the guinea pig model requires 6 months to complete.

Cell-Mediated Immunity and Intracellular Pathogens

As already noted, cell-mediated immunity is central to host defense against TB. In cell-mediated immunity, lymphocytes interact with macrophages – the host cells for intracellular pathogens – in two major ways:

- First, they secrete lymphokines which activate the macrophages, thereby endowing them with the capacity to inhibit the multiplication of and perhaps kill some intracellular pathogens.
- Second, cytotoxic lymphocytes recognize and lyse infected macrophages, thereby depriving them of their preferred niche in the host cell.

Both lymphocyte-macrophage interactions are likely to be important in protective immunity against TB. That being the case, the challenge for the vaccinologist is to identify antigens of the pathogen that allow lymphocytes to carry out their immunoprotective activities.

The Search for Protective Antigens, the Holy Grail of Vaccine Research

The still prevailing view of protective T-cell antigens is that they are rare molecules among many in an organism with some special characteristic that results in unusually high stimulation of T-cells. This view has prompted large scale screening approaches to the identification of such antigens, such as the T-cell western blot assay [2, 3]. However, these approaches have not been particularly fruitful. A major problem with the T-cell western blot assay is that the antigens are not pure and hence are at an unknown point on the dose–response curve. Furthermore, the reactivity of protective antigens can be masked by the presence of other molecules including immunosuppressive molecules. Indeed, in the case of mycobacteria, several structural components have been identified as immunosuppressive, including arabinogalactan and lipoarabinomannan.

Seeking an alternative to screening assays, we opted for a cell biological rather than strictly immunological approach to identification or protective antigens. Instead of concentrating on the intrinsic immunogenicity of a foreign protein, we focused on its "location" in infected host cells, reasoning that it is the availability of a protein for processing and presentation to the immune system which is the overriding determinant of whether or not it is protective. The specific location we focused on was the phagosome of the intracellular pathogen, its home within the mononuclear phagocyte. The antigens found in the phagosome are proteins secreted or otherwise released by the growing pathogen, proteins that we refer to collectively as "extracellular proteins."

Extracellular Proteins as Protective Antigens

We have proposed three hypotheses regarding the role of extracellular proteins of intracellular pathogens in protective immunity [4–9]. First, we hypothesized that extracellular proteins play a key role in inducing cell-mediated and protective immunity against intracellular pathogens during natural infection. Such proteins, by virtue of their release by live organisms into their intracellular compartment in the host cell, are available for proteolytic processing and subsequently presentation on the surface of the infected host cells as peptide fragments bound to the major histocompatibility complex (MHC). The presence of such surface-exposed fragments allows the host immune system to recognize live pathogens sequestered within the host cell and to exert an antimicrobial effect against them. In support of this hypothesis, we have demonstrated that guinea pigs infected with *Legionella pneumophila*, the agent of Legionnaires' disease, or *M. tuberculosis* develop a strong cell-mediated immune response against extracellular proteins of these organisms [4–8, 10].

Second, we hypothesized that immunization of a naive host with extracellular proteins of intracellular pathogens, particularly in the case of pathogens such as *L. pneumophila* and *M. tuberculosis* that reside within a phagosome in host cells, would induce a population of lymphocytes capable of later recognizing and exerting an immune response against infected host cells. This is illustrated in Figure 1.

These lymphocytes would recognize infected host cells by identifying MHC-bound fragments of extracellular proteins displayed on the host-cell surface consequent to the release of the proteins by the intracellular pathogen. It is important that, in a given host, MHC–peptide complexes displayed on the surface of antigen-presenting cells after vaccination be the same or nearly the same as on host cells after infection if vaccine-induced lymphocytes are later to recognize the infected host cells. This should be the case for extracellular proteins since they should enter the exogenous route of antigen processing and presentation, whether delivered to antigen presenting cells via vaccination or released by intracellular pathogens into phagosomes of infected host macrophages. In support of this hypothesis, we demonstrated that (a) immunization of naive hosts with purified extracellular proteins of *L. pneumophila* induces strong protective immunity to a lethal aerosolized dose of *L. pneumophila* in the guinea pig model of Legionnaires' disease [5–7], and (b) immunization of naive hosts with purified extracellular proteins of *M. tuberculosis* induces protective immunity to challenge with aerosolized highly virulent *M. tuberculosis* in the guinea pig model of pulmonary tuberculosis [9].

Finally, we hypothesized that among the extracellular proteins of intracellular pathogens, the major extracellular proteins (that is, those released in greatest abundance) would figure prominently in inducing immunoprotection. We reasoned that such proteins, by virtue of their abundance in the phagosome, would be processed and presented on the surface of host cells most frequently, and would therefore induce a particularly strong cell-mediated immune response. In support of this hypothesis, we demonstrated that immunization of naive hosts with either of two purified major extracellular proteins of *L. pneumophila* induces very strong protective immunity against challenge with that organism [5–7], and subsequently that immunization of naive hosts with the most abundant extracellular protein of *M. tuberculosis*, alone or in combination with other abundant proteins, induces protective immunity against aerosol challenge with that organism [9].

Are the same extracellular proteins of *M. tuberculosis* that are released into broth culture also released into the phagosome in host mononuclear phagocytes? The answer is "Yes." In studies involving the radiolabeling of newly synthesized proteins, we have found that the major extracellular proteins of M. tuberculosis are constitutively expressed in human mononuclear phagocytes [11]. Indeed, the major extracellular proteins are among the major proteins of all types produced by *M. tuberculosis* intracellularly in human mononuclear phagocytes.

Naive Host   **Infected Host**
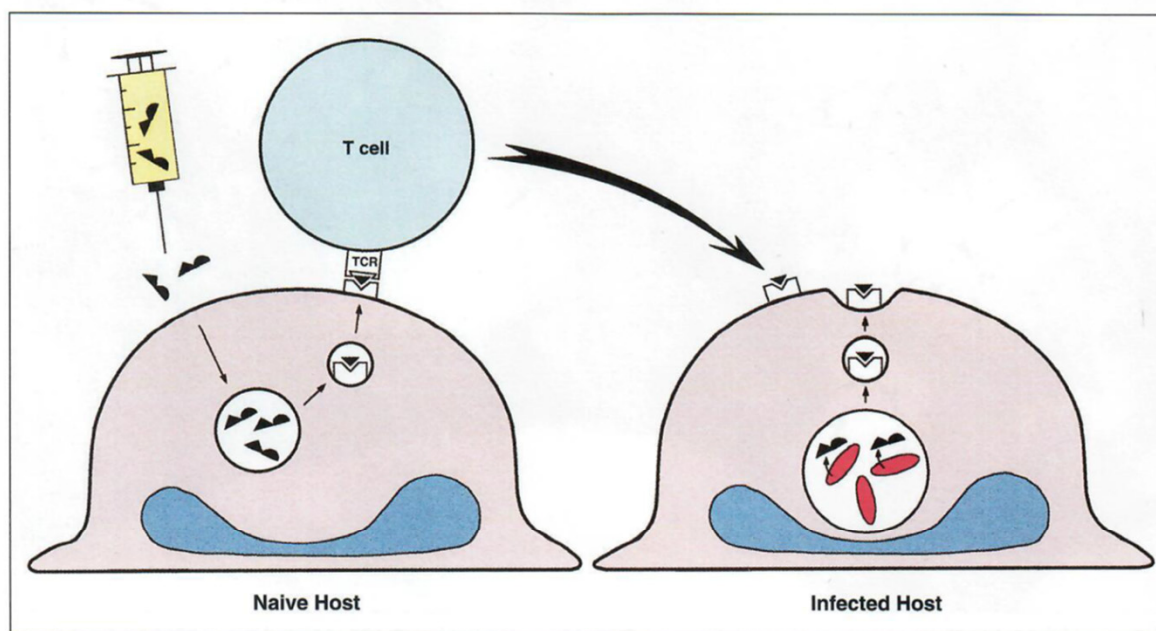
Figure 1. Hypothesis for immunoprotective efficacy of extracellular proteins. Immunization of a naive host with extracellular proteins of *M. tuberculosis* in the presence of an appropriate adjuvant is followed by endocytic uptake of the proteins by antigen-presenting cells (APC). The proteins are proteolytically processed, and fragments of the proteins are bound to MHC molecules. The MHC–peptide complex is presented on the surface of the APC, leading to induction of a population of T-cells that specifically recognize the complex. Later, when the host is infected with *M. tuberculosis*, the bacteria are ingested by mononuclear phagocytes and reside and multiply in a phagosome. There, the bacteria produce and release into the phagosome some of the same extracellular proteins that were present in the vaccine. The proteins are similarly processed, bound to MHC molecules, and presented on the surface of infected mononuclear phagocytes. T-cells previously induced by vaccination recognize the MHC–peptide complex and exert an antimicrobial effect against the infected host cells activating them to resist infection or lysing them to deny the bacteria an intracellular niche.

Using the cryosection immunogold technique, we have confirmed that the major extracellular proteins are released into the phagosome of *M. tuberculosis* in human monocyte-derived macrophages [12, 13] (see Figure 2).

It may be of importance that *M. tuberculosis* resides in a phagosome that has endosomal characteristics, including class II MHC molecules and small amounts of cathepsin D [14, 15]. The phagosome also has class I MHC molecules, perhaps as a result of delayed clearance of the molecules after phagocytosis [14]. However, whether the ready availability of class I and II MHC molecules in the phagosome enhances antigen presentation, or is even necessary, is not clear. *L. pneumophila* resides in a phagosome completely outside the endosomal pathway and devoid of class I and II MHC molecules [14, 16], and yet a strong T-cell-mediated immune response is induced to the major secretory protein that it releases into its phagosome [5, 17]. Presumably, the protein or its proteolytic fragments are transported to another compartment in the cell, such as the class II-containing vesicles (CIIV), for presentation of protein fragments on MHC molecules.

The Acid Test: Animal Challenge

In the absence of a clear-cut correlate of protective immunity, it is ludicrous to refer to an antigen as "protective" unless it has proven itself in an animal challenge experiment. The guinea pig model of pulmonary tuberculosis is the best small animal model because it most closely resembles the disease in humans. In contrast to mice and rats, but like humans, guinea pigs (a) are susceptible to low doses of aerosolized *M. tuberculosis*; (b) exhibit high sensitivity to tuberculin involving a cutaneous delayed-type hypersensitivity reaction characterized by a dense mononuclear cell infiltrate; and (c) display Langhans giant cells and caseation in pulmonary lesions [18]. One difference between guinea pigs and humans is in the incidence of disease following infection. Whereas only 10% of humans develop active disease following infection, 100% of guinea pigs do so. This is an advantage in trials of vaccine efficacy.

Recently, we described the Philippine cynomolgus monkey (*Macaca fasicularis*) model of pulmo-
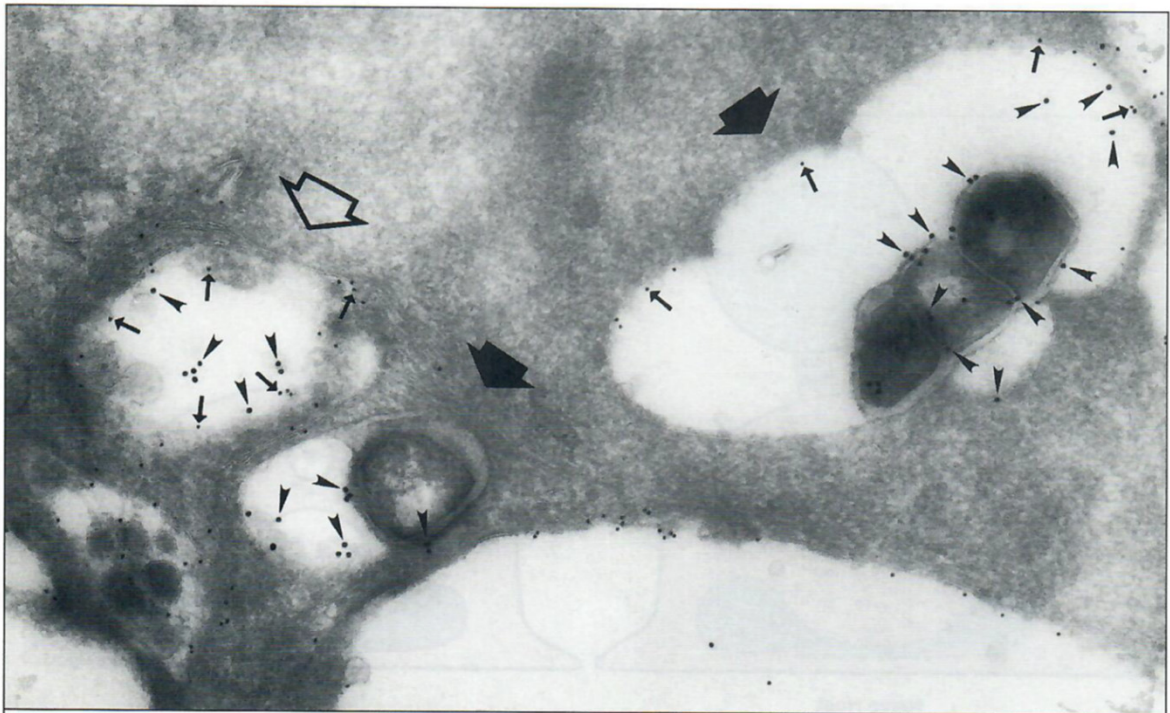
Figure 2. Major extracellular proteins of *M. tuberculosis* are produced by the bacterium and secreted into its phagosome in infected human macrophages. Human monocyte-derived macrophages were infected with the highly virulent Erdman strain of *M. tuberculosis* and stained for the 30/32-kDa complex of major extracellular proteins of *M. tuberculosis* by the cryosection immunogold technique. Two phagosomes containing *M. tuberculosis* (large solid arrows) and a large cytoplasmic vesicle (large open arrow) are present in this portion of the macrophage cytoplasm. The 30/32-kDa complex of proteins, stained with 15-nm-diameter immunogold particles, is present on the cell wall of the bacteria, in the phagosomal space, and the cytoplasmic vesicle outside of the phagosome (small arrowheads). In addition, the late endosomal marker CD63 has been stained with 10-nm-diameter immunogold particles (small arrows). Other host cell markers found on the *M. tuberculosis* phagosomal membrane by this technique include MHC class I and II, the early endosomal marker transferrin receptor and the late endosomal-lysosomal markers LAMP1 and LAMP2. The acid protease cathepsin D also has been found within the phagosome (12, 14, 15). Magnification × 56,000. Reproduced with permission from ref. (12).

nary tuberculosis [19]. This is the first nonhuman primate model of tuberculosis that closely resembles typical disease in humans. In contrast to the rhesus, which rapidly succumbs to fulminant tuberculosis, the Philippine cynomolgus monkey develops a chronic, slowly progressive, localized form of TB akin to the disease in humans. Moreover, these monkeys are often able to contain an infection in a subclinical state. The Philippine cynomolgus monkey model should prove useful for testing vaccines in advanced stages of development.

The Extracellular Protein Vaccine

Using the guinea pig model, we explored the immunoprotective efficacy of the 30-kDa (antigen 85b) major extracellular protein, the most abundant protein released by *M. tuberculosis*, both alone and in combination with other highly abundant extracellular proteins including the 32A (antigen 85A) and 16-kDa proteins (the second and third most abundant extracellular proteins, respectively). These proteins were purified from large-scale cultures of *M. tuberculosis*. After immunizing the animals, we subjected them to a stringent challenge: by the aerosol route because this is the natural route of infection; with the Erdman strain of *M. tuberculosis* because it is the most virulent of the well-characterized strains; and with a high dose of *M. tuberculosis* so that the animals would become ill within a reasonably short time interval.

Our studies showed that immunization with the purified extracellular proteins induced substantial protective immunity. The immunized animals were protected against weight loss, a hallmark of tuberculosis, and against death, and they had fewer live organisms in their lungs and spleen than sham-immunized controls. The 30-kDa protein was effective alone and in combination with other proteins.

Behind Every Great Antigen is an Adjuvant

The ultimate goal of vaccination is the establishment of long-term immunologic memory. Protective antigens do not induce T cell-mediated immunity and long-term memory by themselves. An appropriate adjuvant is required — one that induces a protective as opposed to irrelevant or even counterproductive immune response. In the case of intracellular pathogens, it is likely that a Th1 type of response is important. However, this idea is based largely on studies of *Leishmania* in inbred mice [20, 21], and the extent to which data from that model can be extrapolated to other pathogens and hosts remains to be seen. In any case, our knowledge of adjuvants has unfortunately not yet progressed to the point

where we can select a bottle off the shelf that will yield a Th1 rather than a Th2 or a T-cell helper rather than T-cell killer immune response, or enhance long-term memory. In fact, the only adjuvant approved by the FDA for human use (alum), and most of the adjuvants available for laboratory use were developed because of their capacity to induce humoral rather than T-cell-mediated immune responses. Some of the newer experimental adjuvants, however, such as interleukin 12 (IL-12) [22], show promise as inducers of T-cell-mediated immune responses helpful in combatting intracellular pathogens. The adjuvant dimethyldeoctadecylammonium chloride (DDA) has shown promise in a study of the protective efficacy of crude extracellular proteins in inbred mice [23]. At this point in time, the selection of the best adjuvant is an empirical process that must be determined independently for each immunogen.

Live, Subunit, or DNA Vaccines?

Currently, there are three major approaches to the development of a TB vaccine:

1. Live attenuated mycobacteria;
2. Subunit vaccine; and
3. DNA vaccine.

Live attenuated vaccines represent an old approach to vaccine development with a modern twist – the introduction by recombinant techniques of genes encoding cytokines that it is hoped will enhance the protective immune response [24]. These live vaccines have the advantage of being relatively low cost, but they are likely to suffer the same drawbacks as BCG with respect to safety in immunocompromised individuals and interference with diagnostic tests for TB. It is also exceedingly difficult to standardize a live bacterial vaccine.

A subunit vaccine, such as the one consisting of extracellular proteins described above, has many advantages over a live attenuated vaccine. These are listed in Table 1. Assuming it succeeds in inducing long-lasting protective immunity, the one potential drawback of a subunit vaccine is the somewhat higher cost of a vaccine necessitating purified recombinant proteins and an adjuvant.

DNA vaccines have recently generated a great deal of excitement. In the case of pathogens such as viruses, which inhabit the cytoplasm of host cells, these vaccines offer a mechanism for delivery of antigens via the endogenous route of antigen processing and presentation, thus mimicking the primary route used in natural infection by these pathogens. However, in the case of pathogens such as *M. tuberculosis*, which inhabit a phagosome in host cells and deliver antigens primarily via the readily accessible exogenous route, DNA vaccines offer no such advantage. Although DNA vaccines have been

Table 1. Advantages of a subunit vaccine against tuberculosis

1. It would avoid hazards of a live vaccine and the potential hazards of a DNA vaccine. Because a subunit vaccine consists of only a few molecules, rather than the thousands that comprise a whole organism, it would likely be safe.
2. It would not likely interfere with the PPD skin test, unlike whole organisms.
3. It could be rigorously standardized, unlike whole organisms.
4. It potentially could be combined with childhood vaccines, such as DPT. A live vaccine is more likely to interfere with the immune response to the childhood vaccine.
5. It could avoid irrelevant or immunosuppressive components found in whole organisms.
6. Unlike BCG, it would contain proteins of *M. tuberculosis*.

touted as potentially low cost, it is not yet clear that they will be less costly than recombinant protein vaccines. In addition, DNA vaccines must surmount several major safety concerns, including a theoretical potential for integration into the human genome and insertional mutagenesis, and induction of autoimmunity, immunologic tolerance, or a prolonged allergic reaction to an encoded protein, whose synthesis is not readily terminated. Recently, two DNA vaccines against TB were tested in the mouse model and reported to induce protection comparable to BCG [25, 26]. One of them [25] consists of a gene encoding one of the major extracellular proteins of *M. tuberculosis* (32A) previously included in the subunit vaccine described above.

Conclusions

A new TB vaccine is on the not-too-distant horizon. Because of the many serious problems associated with the use of live vaccines, in all likelihood, the new vaccine will consist of defined molecules selected for their immunoprotective efficacy. Currently, the leading candidates for inclusion in a subunit vaccine are extracellular proteins of *M. tuberculosis*, the only purified proteins thus far demonstrated to be immunoprotective in animal studies. The subunit extracellular protein vaccine developed in our laboratory is at a relatively advanced stage of development. The high level expression and secretion of all the relevant recombinant proteins has been achieved [G. Harth, B.-Y. Lee, and M.A. Horwitz, unpublished data], and the proteins have been demonstrated to be efficacious in the presence of adjuvants used safely in humans [G. Harth, B.J. Dillon, and M.A. Horwitz, unpublished data]. Whether the indirect delivery of these extracellular proteins via a DNA vaccine offers any immunologic or other ad-

vantage over simply directly administrating the proteins with a suitable adjuvant remains to be determined. Thus far only the subunit extracellular protein vaccine has demonstrated to be efficacious in the highly relevant outbred guinea pig model. As for safety: again, only the subunit vaccine lacks major concerns.

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