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(54) Title: ABUNDANT EXTRACELLULAR PRODUCTS AND METHODS FOR THEIR PRODUCTION AND USE

(57) Abstract

Vaccines based on combinations of majorly abundant extracellular products of pathogens and methods for their use and production are presented. The most prevalent or majorly abundant extracellular products of a target pathogen are selected irrespective of their absolute molecular immunogenicity and used as vaccines to stimulate a protective immune response in mammalian hosts against subsequent infection by the target pathogen. The majorly abundant extracellular products may be characterized and distinghuished by their respective N-terminal amino acid sequences. As the vaccines may comprise different combinations of the extracellular products, a broad range effective immunotherapeutic compositions are provided by the present invention. In addition to other infectious agents, the vaccines so produced can be used to stimulate an effective immune response against intracellular pathogens and in particular *Mycobacterium tuberculosis.*

ABUNDANT EXTRACELLULAR PRODUCTS AND METHODS FOR THEIR PRODUCTION AND USE

Cross Reference to a Related Application

This application is a continuation-in-part of co-pending U.S. patent application Ser. No. 156,358 filed on November 23, 1993 and incorporated herein by reference.

Reference to Government

This invention was made with Government support under Grant No. A1-31338 awarded by the Department of Health and Human Services. The Government has certain rights in this invention.

Field of the Invention

The present invention generally relates to immunotherapeutic agents and vaccines against pathogenic organisms such as bacteria, protozoa, viruses and fungus. More specifically, unlike prior art vaccines and immunotherapeutic agents based upon pathogenic subunits or products which exhibit the greatest or most specific molecular immunogenicity, the present invention uses the most prevalent or majorly abundant immunogenic determinants released by a selected pathogen such as *Mycobacterium tuberculosis* to stimulate an effective immune response in mammalian hosts. Accordingly, the acquired immunity and immunotherapeutic activity produced through the present invention is directed to those antigenic markers which are displayed most often on infected host cells during the course of a pathogenic infection without particular regard to the relative or absolute immunogenicity of the administered compound.

Background of the Invention

It has long been recognized that parasitic micro-organisms possess the ability to infect animals thereby causing disease and often the death of the host. Pathogenic agents have been a leading cause of death throughout history and continue to inflict immense suffering.

Though the last hundred years have seen dramatic advances in the prevention and treatment of many infectious diseases, complicated host-parasite interactions still limit the universal effectiveness of therapeutic measures. Difficulties in countering the sophisticated invasive mechanisms displayed by many pathogenic vectors is evidenced by the resurgence of various diseases such as tuberculosis, as well as the appearance of numerous drug resistant strains of bacteria and viruses.

Among those pathogenic agents of major epidemiological concern, intracellular bacteria have proven to be particularly intractable in the face of therapeutic or prophylactic measures. Intracellular bacteria, including the genus *Mycobacterium* and the genus *Legionella*, complete all or part of their life cycle within the cells of the infected host organism rather than extracellularly. Around the world, intracellular bacteria are responsible for millions of deaths each year and untold suffering. Tuberculosis, caused by *Mycobacterium tuberculosis*, is the leading cause of death from infectious disease worldwide, with 10 million new cases and 2.9 million deaths every year. In addition, intracellular bacteria are responsible for millions of cases of leprosy. Other debilitating diseases transmitted by intracellular agents include cutaneous and visceral leishmaniasis, American trypanosomiasis (Chagas disease), listeriosis, toxoplasmosis, histoplasmosis, trachoma, psittacosis, Q-fever, and Legionellosis including Legionnaires' disease. At this time, relatively little can be done to prevent debilitating infections in susceptible individuals exposed to these organisms.

Due to this inability to effectively protect populations from tuberculosis and the inherent human morbidity and mortality caused by tuberculosis, this is one of the most important diseases confronting mankind. More specifically, human pulmonary tuberculosis primarily caused by *M. tuberculosis* is a major cause of death in developing countries. Capable of surviving inside macrophages and monocytes, *M. tuberculosis* may produce a chronic intracellular infection. By concealing itself within the cells primarily responsible for the detection of foreign elements and subsequent activation of the immune system, *M. tuberculosis* is relatively successful in evading the normal defenses of the host organism. These same pathogenic characteristics have heretofore prevented the development of an effective immunotherapeutic agent or vaccine against tubercular infections. At the same time tubercle bacilli are relatively easy to culture and observe under laboratory conditions. Accordingly, *M. tuberculosis* is particularly well suited for demonstrating the principles and advantages of the present invention.

Those skilled in the art will appreciate that the following exemplary discussion of *M. tuberculosis* is in no way intended to limit the scope of the present invention to the treatment of *M. tuberculosis*. Similarly, the teachings herein are not limited in any way to the treatment of tubercular infections. On the contrary, this invention may be used to advantageously provide safe and effective vaccines and immunotherapeutic agents against the immunogenic determinants of any pathogenic agent expressing extracellular products and thereby inhibit the infectious transmission of those organisms.

Currently it is believed that approximately half of the world's population is infected by *M. tuberculosis* resulting in millions of cases of pulmonary tuberculosis annually. While this disease is a particularly acute health problem in the developing countries of Latin America, Africa, and Asia, it is also becoming more prevalent in the first world. In the United States specific populations are at increased risk, especially urban poor, immunocompromised individuals and immigrants from areas of high disease prevalence. Largely due to the AIDS epidemic the incidence of tuberculosis is presently increasing in developed countries, often in the form of multi-drug resistant *M. tuberculosis*.

Recently, tuberculosis resistance to one or more drugs was reported in 36 of the 50 United States. In New York City, one-third of all cases tested in 1991 were resistant to one or more major drugs. Though non-resistant tuberculosis can be cured with a long course of antibiotics, the outlook regarding drug resistant strains is bleak. Patients infected with strains resistant to two or more major antibiotics have a fatality rate of around 50%. Accordingly, a safe and effective vaccine against such varieties of *M. tuberculosis* is sorely needed.

Initial infections of *M. tuberculosis* almost always occur through the inhalation of aerosolized particles as the pathogen can remain viable for weeks or months in moist or dry sputum. Although the primary site of the infection is in the lungs, the organism can also cause infection of the bones, spleen, meninges and skin. Depending on the virulence of the particular strain and the resistance of the host, the infection and corresponding damage to the tissue may be minor or extensive. In the case of humans, the initial infection is controlled in the majority of individuals exposed to virulent strains of the bacteria. The development of acquired immunity following the initial challenge reduces bacterial proliferation thereby allowing lesions to heal and leaving the subject largely asymptomatic but possibly contagious.

When *M. tuberculosis* is not controlled by the infected subject, it often results in the extensive degradation of lung tissue. In susceptible individuals lesions are usually formed in the lung as the tubercle bacilli reproduce within alveolar or pulmonary macrophages. As the organisms multiply, they may spread through the lymphatic system to distal lymph nodes and through the blood stream to the lung apices, bone marrow, kidney and meninges surrounding the brain. Primarily as the result of cell-mediated hypersensitivity responses, characteristic granulomatous lesions or tubercles are produced in proportion to the severity of the infection. These lesions consist of epithelioid cells bordered by monocytes, lymphocytes and fibroblasts. In most instances a lesion or tubercle eventually becomes necrotic and undergoes caseation.

While *M. tuberculosis* is a significant pathogen, other species of the genus *Mycobacterium* also cause disease in animals including man and are clearly within the scope of the present invention. For example, *M. bovis* is closely related to *M. tuberculosis* and is responsible for tubercular infections in domestic animals such as cattle, pigs, sheep, horses, dogs and cats. Further, *M. bovis* may infect humans via the intestinal tract, typically from the ingestion of raw milk. The localized intestinal infection eventually spreads to the respiratory tract and is followed shortly by the classic symptoms of tuberculosis. Another important pathogenic vector of the genus *Mycobacterium* is *M. leprae* which causes millions of cases of the ancient disease leprosy. Other species of this genus which cause disease in animals and man include *M. kansasii, M. avium intracellulare, M. fortuitum, M. marinum, M. chelonei, M. africanum, M. ulcerans, M. microti* and *M. scrofulaceum*. The pathogenic mycobacterial species frequently exhibit a high degree of homology in their respective DNA and corresponding protein sequences and some species, such as *M. tuberculosis* and *M. bovis* are highly related.

For obvious practical and moral reasons, initial work in humans to determine the efficacy of experimental compositions with regard to such afflictions is infeasible. Accordingly, in the early development of any drug or vaccine it is standard procedure to employ appropriate animal models for reasons of safety and expense. The success of implementing laboratory animal models is predicated on the understanding that immunodominant epitopes are frequently active in different host species. Thus, an immunogenic determinant in one species, for example a rodent or guinea pig, will generally be immunoreactive in a different species such as in humans. Only after the appropriate animal models are sufficiently developed will clinical trials in humans be carried out to further demonstrate the safety and efficacy of a vaccine in man.

With regard to alveolar or pulmonary infections by *M. tuberculosis*, the guinea pig model closely resembles the human pathology of the disease in many respects. Accordingly, it is well understood by those skilled in the art that it is appropriate to extrapolate the guinea pig model of this disease to humans and other mammals. As with humans, guinea pigs are susceptible to tubercular infection with low doses of the aerosolized human pathogen *M. tuberculosis*. Unlike humans where the initial infection is usually controlled, guinea pigs consistently develop disseminated disease upon exposure to the aerosolized pathogen, facilitating subsequent analysis. Further, both guinea pigs and humans display cutaneous delayed-type hypersensitivity reactions characterized by the development of a dense mononuclear cell induration or rigid area at the skin test site. Finally, the characteristic tubercular lesions of humans and guinea pigs exhibit similar morphology including the presence of Langhans giant cells. As guinea pigs are more susceptible to initial infection and progression of the disease than humans, any protection conferred in experiments using this animal model provides a strong indication that the same protective immunity may be generated in man or other less susceptible mammals. Accordingly, for purposes of explanation only and not for purposes of limitation, the present invention will be primarily demonstrated in the exemplary context of guinea pigs as the mammalian host. Those skilled in the art will appreciate that the present invention may be practiced with other mammalian hosts including humans and domesticated animals.

Any animal or human infected with a pathogenic vector and, in particular, an intracellular organism presents a difficult challenge to the host immune system. While many infectious agents may be effectively controlled by the humoral response and corresponding production of protective antibodies, these mechanisms are primarily effective only against those pathogens located in the body's extracellular fluid. In particular, opsonizing antibodies bind to extracellular foreign agents thereby rendering them susceptible to phagocytosis and subsequent intracellular killing. Yet this is not the case for other pathogens. For example, previous studies have indicated that the humoral immune response does not appear to play a significant protective role against infections by intracellular bacteria such as *M. tuberculosis*. However, the present invention may generate a beneficial humoral response to the target pathogen and, as such, its effectiveness is not limited to any specific component of the stimulated immune response.

More specifically, antibody mediated defenses seemingly do not prevent the initial infection of intracellular pathogens and are ineffectual once the bacteria are sequestered within the cells of the host. As water soluble proteins, antibodies can permeate the extracellular fluid and blood, but have difficulty migrating across the lipid membranes of cells. Further, the production of opsonizing antibodies against bacterial surface structures may actually assist intracellular pathogens in entering the host cell. Accordingly, any effective prophylactic measure against intracellular agents, such as *Mycobacterium*, should incorporate an aggressive cell-mediated immune response component leading to the rapid proliferation of antigen specific lymphocytes which activate the compromised phagocytes or cytotoxically eliminate them. However, as will be discussed in detail below, inducing a cell-mediated immune response does not equal the induction of protective immunity. Though cell-mediated immunity may be a prerequisite to protective immunity, the production of vaccines in accordance with the teachings of the present invention requires animal based challenge studies.

This cell-mediated immune response generally involves two steps. The initial step, signaling that the cell is infected, is accomplished by special molecules (major histocompatibility or MHC molecules) which deliver pieces of the pathogen to the surface of the cell. These MHC molecules bind to small fragments of bacterial proteins which have been degraded within the infected cell and present them at the surface of the cell. Their presentation to T-cells stimulates the immune system of the host to eliminate the infected host cell or induces the host cell to eradicate any bacteria residing within.

Unlike most infectious bacteria *Mycobacterium*, including *M. tuberculosis*, tend to proliferate in vacuoles which are substantially sealed off from the rest of the cell by a membrane. Phagocytes naturally form these protective vacuoles making them particularly susceptible to infection by this class of pathogen. In such vacuoles the bacteria are effectively protected from degradation, making it difficult for the immune system to present integral bacterial components on the surface of infected cells. However, the infected cell's MHC molecules will move to the vacuole and collect any free (released) bacterial products or move to other sites in the host cell to which the foreign extracellular bacterial products have been transported for normal presentation of the products at the cell surface. As previously indicated, the presentation of the foreign bacterial products will provoke the proper response by the host immune system.

The problems intracellular pathogens pose for the immune system also constitute a special challenge to vaccine development. Thus far, the production of an effective vaccine against *Mycobacterium* infections and, in particular, against *M. tuberculosis* has eluded most researchers. At the present time the only widely available vaccine against intracellular pathogens is the live attenuated vaccine BCG, an avirulent strain of *M. bovis*, which is used as a prophylactic measure against the tubercle bacillus. Yet in 1988, extensive World Health Organization studies from India determined that the efficacy of the best BCG vaccines was so slight as to be unmeasurable. Despite this questionable efficacy, BCG vaccine has been extensively employed in high incidence areas of tuberculosis throughout the world. Complicating the matter even further individuals who have been vaccinated with BCG will often develop sensitivity to tuberculin which negates the usefulness of the most common skin test for tuberculosis screening and control.

Another serious problem involving the use of a live, attenuated vaccine such as BCG is the possibility of initiating a life-threatening disease in immunocompromised patients. These vaccines pose a particular risk for persons with depressed cell-mediated immunity because of their diminished capacity to fight a rapidly proliferating induced infection. Such individuals include those weakened by malnourishment and inferior living conditions, organ transplant recipients, and persons infected with HIV. In the case of BCG vaccine, high risk individuals also include those suffering from lung disorders such as emphysema, chronic bronchitis, pneumoconiosis, silicosis or previous tuberculosis. Accordingly, the use of attenuated vaccines is limited in the very population where they have the greatest potential benefit.

The use of live attenuated vaccines may also produce other undesirable side effects. Because live vaccines reproduce in the recipient, they provoke a broader range of antibodies and a less directed cell-mediated immune response than noninfectious vaccines. Often this shotgun approach tends to occlude the immune response directed at the molecular structures most involved in cellular prophylaxis. Moreover, the use of live vaccines with an intact membrane may induce opsonizing antibodies which prepare a foreign body for effective phagocytosis. Thus, upon host exposure to virulent strains of the target organism, the presence of such antibodies could actually enhance the uptake of non-attenuated pathogens into host cells where they can survive and multiply. Further, an attenuated vaccine contains thousands of different molecular species and consequently is more likely to contain a molecular species that is toxic or able to provoke an adverse immune response in the patient. Other problems with live vaccines include virulence reversion, natural spread to contacts, contaminating viruses and viral interference, and difficulty with standardization.

Similarly, noninfectious vaccines, such as killed organisms or conventional second generation subunit vaccines directed at strongly antigenic membrane bound structures, are limited with respect to the inhibition of intracellular bacteria. Like attenuated vaccines, killed bacteria provoke an indiscriminate response which may inhibit the most effective prophylactic determinants. Further, killed vaccines still present large numbers of potentially antigenic structures to the immune system thereby increasing the likelihood of toxic reactions or opsonization by the immune system. Traditional subunit vaccines incorporating membrane bound structures, whether synthesized or purified, can also induce a strong opsonic effect facilitating the entry of the intracellular pathogen into phagocytes in which they multiply. By increasing the rate of bacterial inclusion, killed vaccines directed to intracellular surface antigens may increase the relative virulence of the pathogenic agent. Thus, conventional attenuated or killed vaccines directed against strongly antigenic bacterial surface components may be contraindicated in the case of intracellular pathogens.

In order to circumvent the problems associated with the use of traditional vaccines, developments have been made using extracellular proteins or their immunogenic analogs to stimulate protective immunity against specific intracellular pathogens. For example, this inventor's U.S. Patent No. 5,108,745, issued April 28, 1992 discloses vaccines and methods of producing protective immunity against *Legionella pneumophila* and *M. tuberculosis* as well as other intracellular pathogens. These prior art vaccines are broadly based on extracellular products originally derived from proteinaceous compounds released extracellularly by the pathogenic bacteria into broth culture *in vitro* and released extracellularly by bacteria within infected host cells *in vivo*. As disclosed therein, these vaccines are selectively based on the identification of extracellular products or their analogs which stimulate a strong immune response against the target pathogen in a mammalian host.

More specifically, these prior art candidate extracellular proteins were screened by determining their ability to provoke either a strong lymphocyte proliferative response or a cutaneous delayed-type hypersensitivity response in mammals which were immune to the pathogen of interest. Though this disclosed method and associated vaccines avoid many of the drawbacks inherent in the use of traditional vaccines, conflicting immunoresponsive results due to cross-reactivity and host variation may complicate the selection of effective immunizing agents. Thus, while molecular immunogenicity is one indication of an effective vaccine, other factors may complicate its use in eliciting an effective immune response *in vivo*.

More importantly, it surprisingly was discovered that, particularly with respect to *M. tuberculosis*, conventional prior art methods for identifying effective protective immunity inducing vaccines were cumbersome and potentially ineffective. For example, SDS-PAGE analysis of bulk *M. tuberculosis* extracellular protein followed by conventional Western blot techniques aimed at identifying the most immunogenic of these extracellular components produced inconsistent results. Repeated testing failed to identify which extracellular product would produce the strongest immunogenic response and, consistent with prior art thinking, thereby function as the most effective vaccine. Many of the extracellular products of *M. tuber-*

culosis are well known in the art, having been identified and, in some cases, sequenced. Further, like any foreign protein, it can be shown that these known compounds induce an immune response. However, nothing in the art directly indicates that any of these known compounds will induce protective immunity as traditionally identified.

Accordingly, it is a principal object of the present invention to provide vaccines or immunotherapeutic agents and methods for their production and use in mounting an effective immune response against infectious bacterial pathogens which do not rely upon traditional vaccine considerations and selection techniques based upon highly specific, strongly immunogenic operability.

It is another object of the present invention to provide vaccines or immunotherapeutic agents and methods for their use to impart acquired immunity in a mammalian host against intracellular pathogens including *M. tuberculosis, M. bovis, M. kansasii, M. avium-intracellulare, M. fortuitum, M. chelonei, M. marinum, M. scrofulaceum, M. leprae, M. africanum, M. ulcerans* and *M. microti*.

It is an additional object of the present invention to provide easily produced vaccines and immunotherapeutic agents exhibiting reduced toxicity relative to killed or attenuated vaccines.

Summary of the Invention

The present invention accomplishes the above-described and other objects by providing compounds for use as vaccines and/or immunotherapeutic agents and methods for their production to generate protective or therapeutic immune responses in mammalian hosts against infection by pathogens. In a broad aspect, the invention provides the means to induce a protective or therapeutic immune response against infectious vectors producing extracellular compounds. While the compounds of the present invention are particularly effective against pathogenic bacteria, they may be used to generate a protective or therapeutic immune response to any pathogen producing majorly abundant extracellular products.

For purposes of the present invention, the term "majorly abundant" should be understood as a relative term identifying those extracellular products released in the greatest quantity by the pathogen of interest. For example, with respect to *M. tuberculosis* grown under various conditions of culture to an optical density of approximately 0.5, one skilled in the art should expect to obtain on the order of 10 μg/L or more of a majorly abundant extracellular product. Thus, out of the total exemplary 4 mg/L total output of extracellular product for *M. tuberculosis* grown under normal or heat shock conditions, approximately fifteen to twenty (alone or in combination) of the one hundred or so known extracellular products will constitute approximately ninety percent of the total quantity. These are the majorly abundant extracellular products contemplated as being within the scope of the present invention and are readily identifiable as the broad bands appearing in SDS/PAGE gels. In addition, the extracellular products of interest may further be characterized and differentiated by amino acid sequencing. The remaining extracellular products are minor. Those skilled in the art will also appreciate that the relative quantitative abundance of specific major extracellular products may vary depending upon conditions of culture. However, in most cases, the identification of an individual majorly abundant extracellular product will not change.

Accordingly, the present invention may be used to protect a mammalian host against infection by viral, bacterial, fungal or protozoan pathogens. It should be noted that in some cases, such as in viral infections, the majorly abundant extracellular products may be generated by the infected host cell. While active against all microorganisms releasing majorly abundant extracellular products, the vaccines and methods of the present invention are particularly effective in generating protective immunity against intracellular pathogens, including various species and serogroups of the genus *Mycobacterium*. The vaccines of the present invention are also effective as immunotherapeutic agents for the treatment of existing disease conditions.

Surprisingly, it has been found by this inventor that immunization with the most or majorly abundant products released extracellularly by bacterial pathogens or their immunogenic analogs can provoke an effective immune response irrespective of the absolute immunogenicity of the administered compound. Due to their release from the organism and hence their availability to host molecules involved in antigen processing and presentation and due to their naturally high concentration in tissue during infection, the majorly abundant extracellular products of a pathogenic agent are processed and presented to the host immune system more often than other bacterial components. In the case of intracellular pathogens, the majorly abundant extracellular products are the principal immunogenic determinants presented on the surface of the infected host cells and therefore exhibit a greater presence in the surrounding environment. Accordingly, acquired immunity against the majorly abundant extracellular products of a pathogenic organism allows the host defense system to swiftly detect pathogens sequestered inside host cells and effectively inhibit them.

More particularly, the principal or majorly abundant products released by pathogenic bacteria appear to be processed by phagocytes and other host immune system mechanisms at a greater rate than less prevalent or membrane bound pathogenic components regardless of their respective immunogenic activity or specificity. This immunoprocessing disparity is particularly significant when the pathogenic agent is an intracellular bacteria sequestered from normal immune activity. By virtue of their profuse and continual presentation to the infected host's immune system, the most prevalent bacterial extracellular products or their immunogenic analogs provoke a vigorous immune response largely irrespective of their individual molecular immunogenic characteristics.

Majorly abundant extracellular products are the principal constituents of proteins and other molecular entities which are released by the target pathogen into the surrounding environment. Current research indicates that in some instances a single majorly abundant extracellular product may comprise up to 40% by weight of the products released by a microorganism. More often, individual majorly abundant extracellular products account for between from about 0.5% to about 25% of the total products released by the infectious pathogen. Moreover, the top five or six majorly abundant extracellular products may be found to comprise between 60% to 70% of the total mass released by a microorganism. Of course those skilled in the art will appreciate that the relative levels of extracellular products may fluctuate over time as can the absolute or relative quantity of products released. For example, pH, oxidants, osmolality, heat and other conditions of stress on the organism, stage of life cycle, reproduction status and the composition of the surrounding environment may alter the composition and quantity of products released. Further, the absolute and relative levels of extracellular products may differ greatly from species to species and even between strains within a species.

In the case of intracellular pathogens extracellular products appear to expand the population of specifically immune lymphocytes capable of detecting and exerting an antimicrobial effect against macrophages containing live bacteria. Further, by virtue of their repeated display on the surface of infected cells, the majorly abundant or principal extracellular products function as effective antigenic markers. Accordingly, pursuant to the teachings of the present invention, vaccination and the inducement of protective immunity directed to the majorly abundant extracellular products of a pathogenic bacteria or their immunogenically equivalent determinants, prompts the host immune system to mount a rapid and efficient immune response with a strong cell-mediated component when subsequently infected by the target pathogen.

In direct contrast to prior art immunization activities which have primarily been focused on the production of vaccines and the stimulation of immune responses based upon the highly specific molecular antigenicity of individual screened pathogen components, the present invention advantageously exploits the relative abundance of bacterial extracellular products or their immunogenic analogs (rather than their immunogenic specificities) to establish or induce protective immunity with compounds which may actually exhibit lower immunogenic specificity than less prevalent extracellular products. For the purposes of this disclosure an immunogenic analog is any molecule or compound sufficiently analogous to at least one majorly abundant extracellular product expressed by the target pathogen, or any fraction thereof, to have the capacity to stimulate a protective immune response in a mammalian host upon subsequent infection by the target pathogen. In short, the vaccines of the present invention are identified or produced by selecting the majorly abundant product or products released extracellularly by a specific pathogen (or molecular analogs capable of stimulating a substantially equivalent immune response) and isolating them in a relatively pure form. The desired prophylactic immune response to the target pathogen may then be elicited by formulating one or more of the isolated immunoreactive products using techniques well known in the art and immunizing a mammalian host prior to infection by the target pathogen.

It is anticipated that the present invention will consist of at least one, two or, possibly even several well defined immunogenic determinants. As a result, the present invention produces consistent, standardized vaccines which may be developed, tested and administered with relative ease and speed. Further, the use of a few well defined molecules corresponding to the majorly abundant secretory or extracellular products greatly reduces the risk of adverse side effects associated with conventional vaccines and eliminates the possible occlusion of effective immunogenic markers. Similarly, because the present invention is not an attenuated or a killed vaccine the risk of infection during production, purification or upon administration is effectively eliminated. As such, the vaccines of the present invention may be administered safely to immunocompromised individuals, including asymptomatic tuberculosis patients and those infected with HIV. Moreover, as the humoral immune response is directed exclusively to products released by the target pathogen, there is little chance of generating a detrimental opsonic immune component. Accordingly, the present invention allows the stimulated humoral response to assist in the elimination of the target pathogen from antibody susceptible areas.

Another beneficial aspect of the present invention is the ease by which the vaccines may be harvested or produced and subsequently purified. For example, the predominantly abundant extracellular products may be obtained from cultures of the target pathogen, including *M. tuberculosis* or *M. bovis*, with little effort. As the desired compounds are released into the media during growth, they can readily be separated from the intrabacterial and membrane-bound components of the target pathogen utilizing conventional techniques. More preferably, the desired immunoreactive constituents of the vaccines of the present invention may be produced and purified from genetically engineered organisms into which the genes expressing the specific extracellular products of *M. tuberculosis*, *M. bovis*, *M. leprae* or any other pathogen of interest have been cloned. As known in the art, such engineered organisms can be modified to produce higher levels of the selected extracellular products or modified immunogenic analogs. Alternatively, the immunoprotective products, portions thereof or analogs thereof, can be chemically synthesized using techniques well known in the art. Whatever production source is employed, the immunogenic components of the predominant or majorly abundant extracellular products may be separated and subsequently formulated into deliverable vaccines using common biochemical procedures such as fractionation, chromatography or other purification methodology and conventional formulation techniques.

For example, in an exemplary embodiment of the present invention the target pathogen is *M. tuberculosis* and the majorly abundant products released extracellularly by *M. tuberculosis* into broth culture are separated from other bacterial components and used to elicit an immune response in mammalian hosts. Individual proteins or groups of proteins are then utilized in animal based challenge experiments to identify those which induce protective immunity making them suitable for use as vaccines in accordance with the teachings of the present invention. More specifically, following the growth and harvesting of the bacteria, by virtue of their physical abundance the principal extracellular products are separated from intrabacterial and other components through centrifugation and filtration. If desired, the resultant bulk filtrate is then subjected to fractionation using ammonium sulfate precipitation with subsequent dialysis to give a mixture of extracellular products, commonly termed EP. Solubilized extracellular products in the dialyzed fractions are then purified to substantial homogeneity using suitable chromatographic techniques as known in the art and as described more fully below.

These exemplary procedures result in the production of fourteen individual proteinaceous major extracellular products of *M. tuberculosis* having molecular weights ranging from 110 kilo Daltons (KD) to 12 KD. Following purification each individual majorly abundant extracellular product exhibits one band corresponding to its respective molecular weight when subjected to polyacrylamide gel electrophoresis thereby allowing individual products or groups of products corresponding to the majorly abundant extracellular products to be identified and prepared for use as vaccines in accordance with the teachings of the present invention. The purified majorly abundant extracellular products may further be characterized and distinguished by determining all or part of their respective amino acid sequences using techniques common in the art. Sequencing may also provide information regarding possible structural relationships between the majorly abundant extracellular products.

Subsequently, immunization and the stimulation of acquired immunity in a mammalian host system may be accomplished through the teachings of the present invention utilizing a series of subcutaneous or intradermal injections of these purified extracellular products over a course of time. For example, injection with a purified majorly abundant bacterial extracellular product or products in incomplete Freund's adjuvant followed by a second injection in the same adjuvant approximately three weeks later can be used to elicit a protective response upon subsequent challenge with the virulent pathogen. Other exemplary immunization protocols within the scope and teachings of the present invention may include a series of three or four injections of purified extracellular product or products or their analogs in Syntex Adjuvant Formulation (SAF) over a period of time. While a series of injections may generally prove more efficacious, the single administration of a selected majorly abundant extracellular product or its immunogenic subunits or analogs can impart the desired immune response and is contemplated as being within the scope of the present invention as well.

Such exemplary protocols can be demonstrated using art accepted laboratory models such as guinea pigs. For example, as will be discussed in detail, immunization of several guinea pigs with a combination of five majorly abundant extracellular products (purified from *M. tuberculosis* as previously discussed) was accomplished with an immunization series of three injections of the bacterial products in SAF adjuvant with corresponding sham-immunization of control animals. Exemplary dosages of each protein ranged from 100 μg to 2 μg. Following the last vaccination all of the animals were simultaneously exposed to an infectious and potentially lethal dose of aerosolized *M. tuberculosis* and monitored for an extended period of time. The control animals showed a significant loss in weight when compared with the animals immunized with the combination of the majorly abundant extracellular products of *M. tuberculosis*. Moreover, half of the control animals died during the observation period while none of the immunized animals succumbed to tuberculosis. Autopsies conducted after this experiment revealed that the non-immunized control animals had significantly more colony forming units (CFU) and corresponding damage in their lungs and spleens than the protected animals. Seventeen additional combinations of purified majorly abundant extracellular products provided immunoprophylaxis when tested, thereby demonstrating the scope of the present invention and broad range of vaccines which may be formulated in accordance with the teachings thereof.

However, it should be emphasized that the present invention is not restricted to combinations of secretory or extracellular products. For example, several alternative experimental protocols demonstrate the capacity of a single abundant extracellular product to induce mammalian protective immunity in accordance with the teachings of the present invention. In each experiment guinea pigs were immunized with a single majorly abundant extracellular product purified from *M. tuberculosis* EP using the chromatography protocols detailed herein. In one example the animals were vaccinated in multiple experiments with an adjuvant composition containing a purified abundant secretory product having a molecular weight corresponding to 30 KD. In another example of the present invention, different guinea pigs were vaccinated with an adjuvant composition containing an abundant extracellular product isolated from *M. tuberculosis* having a molecular weight corresponding to 71 KD. Following their respective immunizations both sets of animals and the appropriate controls were exposed to lethal doses of aerosolized *M. tuberculosis* to determine vaccine effectiveness.

More particularly, in one experiment six guinea pigs were immunized with 100 μg of 30 KD protein in SAF on three occasions spread over a period of six weeks. Control animals were simultaneously vaccinated with corresponding amounts of a bulk preparation of extracellular proteins (EP) or buffer. Three weeks after the final vaccination, the animals were challenged with an aerosolized lethal dose of *M. tuberculosis* and monitored for a period of 14 weeks. The 30 KD immunized guinea pigs and those immunized with the bulk extracellular preparation had survival rates of 67% and 50% respectively (illustrating the unexpectedly superior performance of the majorly abundant extracellular product versus EP), while the sham-immunized animals had a survival rate of only 17%. Upon termination of the experiment the animals were sacrificed and examined for viable tubercle bacilli. Unsurprisingly, the non-immunized animal showed markedly higher concentrations of *M. tuberculosis* in the lungs and spleen.

Similar experiments were performed on those animals vaccinated with 71 KD protein. In one experiment six guinea pigs were vaccinated with an SAF adjuvant composition containing 100 μg purified 71 KD protein two times over a period of three weeks. Other animals were similarly immunized with a bulk preparation of unpurified extracellular proteins or EP for use as a positive control and with buffer for use as a negative control. Following exposure to lethal doses of aerosolized tubercle bacilli the weight of the guinea pigs was monitored for a period of 6 months. Once again the animals immunized with the purified form of the abundant extracellular product developed protective immunity with respect to the virulent *M. tuberculosis*. By the end of that period the buffer immunized animals showed a significant loss in weight when compared with the immunized animals. Further, while the positive controls and 71 KD immunized animals had survival rates of 63% and 50% respectively, the non-immunized animals all died before the end of the observation period.

It is important to note that the formulation of the vaccine is not critical to the present invention and may be optimized to facilitate administration. Solutions of the purified immunogenic determinants derived from the majorly abundant pathogenic extracellular products may be administered alone or in combination in any manner designed to generate a protective immune response. The purified protein solutions may be delivered alone, or formulated with an adjuvant before being administered. Specific exemplary adjuvants used in the instant invention to enhance the activity of the selected immunogenic determinants are SAF, adjuvants containing Monophosphoryl Lipid A, Freund's incomplete adjuvant and Freund's complete adjuvant containing killed bacteria. Additional adjuvants that may be useful in the present invention are water-in-oil emulsions, mineral salts (for example, alum), nucleic acids, block polymer surfactants, and microbial cell walls (peptido glycolipids). While not limiting the scope of the invention it is believed that adjuvants may magnify immune responses due to the slow release of antigens from the site of injection.

Other objects, features and advantages of the present invention will be apparent to those skilled in the art from a consideration of the following detailed description of preferred exemplary embodiments thereof taken in conjunction with the figures which will first be described briefly.

Brief Description of the Drawings

Fig. 1 is a representation of 4 coomassie blue stained gels, labeled 1A to 1D, illustrating the purification of exemplary majorly abundant extracellular products of *M. tuberculosis* as identified by sodium deodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Fig. 2 is a tabular representation identifying the five N-terminal amino acids of twelve exemplary majorly abundant extracellular products of *M. tuberculosis* and the apparent molecular weight for fourteen such products.

Fig. 3 is a tabular representation of the extended N-terminal amino acid sequence of three exemplary majorly abundant secretory products of *M. tuberculosis* which were not distinguished by the five N-terminal amino acids shown in Fig. 2.

Fig. 4 is a graphical comparison of the survival rate of guinea pigs immunized with exemplary purified majorly abundant 30 KD secretory product of *M. tuberculosis* versus positive controls immunized with a prior art bulk preparation of extracellular proteins and non-immunized negative controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Fig. 5 is a graphical comparison of mean guinea pig body weight of animals immunized with purified majorly abundant 71 KD extracellular product versus positive controls immunized with a prior art bulk preparation of extracellular proteins from *M. tuberculosis* and non-immunized negative controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Fig. 6 is a graphical comparison of the survival rate of guinea pigs immunized in Fig. 5 with exemplary majorly abundant purified 71 KD extracellular product of *M. tuberculosis* versus positive controls immunized with a prior art bulk preparation of extracellular proteins from *M. tuberculosis* and non-immunized negative controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Fig. 7 is a graphical comparison of mean guinea pig body weight of animals immunized with exemplary purified majorly abundant 71 KD extracellular product and non-immunized negative controls following exposure to an aerosolized lethal dose of *M. tuberculosis* in a second, separate experiment.

Figs. 8A and 8B are graphical comparisons of lymphocyte proliferative responses to exemplary purified majorly abundant 71 KD extracellular product in PPD+ (indicative of infection with M. tuberculosis) and PPD- human subjects. Fig. 8A is a graph of the values measured at 2 days after incubation of lymphocytes with this antigen while Fig. 8B is a graph of the values measured at 4 days after incubation.

Fig. 9 is a graphical comparison of mean guinea pig body weight of animals immunized with vaccine comprising a combination of extracellular products produced according to the teachings of the present invention and non-immunized controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Fig. 10 is a graphical comparison of mean guinea pig body weight of animals immunized with three different dosages of a vaccine comprising a combination of extracellular products produced according to the teachings of the present invention and non-immunized controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Fig. 11 is a graphical comparison of mean guinea pig body weight of animals immunized with vaccines comprising six different combinations of extracellular products produced according to the teachings of the present invention and non-immunized controls following exposure to an aerosolized lethal dose of *M. tuberculosis*.

Detailed Description

The present invention is directed to compounds and methods for their production and use against pathogenic organisms as vaccines and immunotherapeutic agents. More specifically, the present invention is directed to the production and use of majorly abundant extracellular products released by pathogenic organisms or their immunogenic analogs as vaccines or immunotherapeutic agents and to associated methods for generating protective immunity in mammalian hosts against infection. These compounds will be referred to as vaccines throughout this application for purposes of simplicity.

In exemplary embodiments, illustrative of the teachings of the present invention, the majorly abundant extracellular products of *M. tuberculosis* were distinguished and subsequently purified. Guinea pigs were immunized with purified forms of these majorly prevalent extracellular products with no determination of the individual product's specific molecular immunogenicity. Further, the exemplary immunizations were carried out using the purified extracellular products alone or in combination and with various dosages and routes of administration. Those skilled in the art will recognize that the foregoing strategy can be utilized with any pathogenic organism or bacteria to practice the method of the present invention and, accordingly, the present invention is not specifically limited to vaccines and methods directed against *M. tuberculosis*.

In these exemplary embodiments, the majorly abundant extracellular products of *M. tuberculosis* were separated and purified using column chromatography. Determination of the relative abundance and purification of the extracellular products was accomplished using polyacrylamide gel electrophoresis. Following purification of the vaccine components, guinea pigs were vaccinated with the majorly abundant extracellular products alone or in combination and subsequently challenged with *M. tuberculosis*. As will be discussed in detail, in addition to developing the expected measurable responses to these extracellular products following immunization, the vaccines of the present invention unexpectedly conferred an effective immunity in these laboratory animals against subsequent lethal doses of aerosolized *M. tuberculosis*.

While these exemplary embodiments used purified forms of the extracellular products, those skilled in the art will appreciate that the present invention may easily be practiced using immunogenic analogs which are produced through recombinant means or other forms of chemical synthesis using techniques well known in the art. Further, immunogenic analogs, homologs or selected segments of the majorly abundant extracellular products may be employed in lieu of the naturally occurring products within the scope and teaching of the present invention.

A further understanding of the present invention will be provided to those skilled in the art from the following non-limiting examples which illustrate exemplary protocols for the identification, isolation, production and use of majorly abundant extracellular products (alone and in combination) as vaccines.

Example 1

Isolation and Production of Bulk Extracellular Proteins (EP) from *Mycobacterium tuberculosis*

M. tuberculosis Erdman strain (ATCC 35801) was obtained from the American Tissue Culture Collection (Rockville, Md.). The lyophilized bacteria were reconstituted in Middlebrook 7H9 culture medium (Difco Laboratories, Detroit, Mich.) and maintained on Middlebrook 7H11 agar. 7H11 agar was prepared using Bacto Middlebrook 7H10 agar (Difco), OADC Enrichment Medium (Difco), 0.1% casein enzymatic hydrolysate (Sigma), and glycerol as previously described by Cohn (Cohn, M.L., *Am. Rev. Respir. Dis.* 98:295-296) and incorporated herein by reference. Following sterilization by autoclaving, the agar was dispensed into bacteriologic petri dishes (100 by 15 mm) and allowed to cool.

M. tuberculosis was then plated using sterile techniques and grown at 37°C in 5% CO_2-95% air, 100% humidity.

After culture on 7H11 for 7 days, the colonies were scraped from the plates, suspended in 7H9 broth to 10^8 CFU/ml and aliquoted into 1.8-ml Nunc cryotubes (Roskilde, Denmark). Each liter of the broth was prepared by rehydrating 4.7 g of Bacto Middlebrook 7H9 powder with 998 ml of distilled water, and 2 ml of glycerol (Sigma Chemical Co., St. Louis, Mo.) before adjusting the mixture to a pH value of 6.75 and autoclaving the broth for 15 min at 121°C. The aliquoted cells were then slowly frozen and stored at -70°C. Cells stored under these conditions remained viable indefinitely and were used as needed.

Bulk extracellular protein (EP) preparations were obtained from cultures of *M. tuberculosis* grown in the Middlebrook 7H9 broth made as above. Following reconstitution, 150 ml aliquots of the broth were autoclaved for 15 min at 121°C and dispensed into vented Co-star 225 cm^2 tissue culture flasks. *M. tuberculosis* cells stored at -70°C as described in the previous paragraph were thawed and used to inoculate 7H11 agar plates. After culture for 7 days, the colonies were scraped from the plates, suspended in a few ml of 7H9 broth, and sonicated in a water bath to form a single cell suspension. The *M. tuberculosis* cells were suspended in the sterile 150 ml aliquots at an initial optical density of 0.05, as determined by a Perkin-Elmer Junior model 35 spectrophotometer (Norwalk, Conn). The cells were then incubated at 37°C in 5% CO_2-95% air for 3 weeks until the suspension showed an optical density of 0.4 to 0.5. These cultures were used as stock bottles for subsequent cultures also in 7H9 broth. The stock bottles were sonicated in a water bath to form a single cell suspension. The *M. tuberculosis* cells were then diluted in 7H9 broth to an initial optical density of 0.05 and incubated at 37°C in 5% CO^2-95% air for $2\frac{1}{2}$ to 3 weeks until the suspension showed an optical density of 0.4 to 0.5. Culture supernatant was then decanted and filter sterilized sequentially through 0.8 μm and 0.2 μm low-protein-binding filters (Gelman Sciences Inc., Ann

Arbor, Mich.). The filtrate was then concentrated approximately 35 fold in a Filtron Minisette with an Omega membrane having a 10 KD cutoff and stored at 4°C. Analysis of the bulk extracellular protein preparation by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a protein composition with multiple bands. Bulk extracellular protein mixture (EP) was prepared by obtaining a 40-95% ammonium sulfate cut of the culture filtrate.

Example 2

Purification of Principal Majorly Abundant Extracellular Products of *Mycobacterium tuberculosis*

Ammonium sulfate (grade I, Sigma) was added to the sterile culture filtrate of Example 1 in concentrations ranging from 10% to 95% at 0°C and gently stirred to fractionate the proteins. The suspension was then transferred to plastic bottles and centrifuged in a swinging bucket rotor at 3,000 rpm on a RC3B Sorvall Centrifuge to pellet the resulting precipitate. The supernatant fluid was decanted and, depending on the product of interest, the supernatant fluid or pellet was subjected to further purification. When the product of interest was contained in the supernatant fluid a second ammonium sulfate cut was executed by increasing the salt concentration above that of the first cut. After a period of gentle stirring the solution was then centrifuged as previously described to precipitate the desired product and the second supernatant fluid was subjected to further purification.

Following centrifugation, the precipitated proteins were resolubilized in the appropriate cold buffer and dialyzed extensively in a Spectrapor dialysis membrane (Spectrum Medical Industries, Los Angeles, California) with a 6,000 to 8,000 molecular weight cut-off to remove the salt. Extracellular protein concentration was determined by a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Illinois) and fraction components were determined using SDS-PAGE. The fractions were then applied to chromatography columns for further purification.

Using the general scheme outlined immediately above fourteen extracellular products were purified from the bulk extracellular protein filtrate obtained by the process detailed in Example 1. The exact ammonium sulfate precipitation procedure and chromatography protocol is detailed below for each extracellular product isolated.

A. 110 KD Extracellular Product

1. A 50-100% ammonium sulfate precipitate was obtained as discussed above.
2. The resolubilized precipitate was dialyzed and applied to a DEAE Sepharose CL-6B or QAE Sepharose ion exchange column in column buffer consisting of 10% sorbitol, 10 mM potassium phosphate, pH 7, 5 mM 2-mercaptoethanol, and 0.2 mM EDTA and eluted with a sodium chloride gradient. Fractions containing 110 KD protein elute at approximately 550 mM salt and were collected.
3. Collected fractions were applied to S200 Sepharose size fractionation column in PBS (phosphate buffered saline) buffer. The protein eluted as a homogeneous 110 KD protein.

B. 80 KD Extracellular Product

1. The 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded and the 25-60% ammonium sulfate cut (overnight at 0°C) was retained as discussed above.
2. A DEAE CL-6B column (Pharmacia) was charged with 25mM Tris, pH 8.7 containing 1M NaCl and equilibrated with 25mM Tris, pH 8.7, 10mM NaCl and the protein sample was dialyzed against 25mM Tris, pH 8.7, 10mM NaCl and applied to the column. The column was washed overnight with the same buffer. A first salt gradient of 10mM to 200 mM

NaCl in 25mM Tris, pH 8.7 was run through the column to elute other proteins. A second salt gradient (200 to 300 mM NaCl) was run through the column and the 80 KD protein eluted at approximately 275 mM NaCl.

3. A Q-Sepharose HP column was charged with 25mM Tris, pH 8.7, 1M NaCl and re-equilibrated to 25mM Tris, pH 8.7, 10mM NaCl. The protein sample was dialyzed against 25mM Tris, ph 8.7, 10mM NaCl and applied to the column. The column was washed in the same buffer and then eluted with 200-300 mM NaCl in 25mM Tris, pH 8.7.
4. Fractions containing the 80 KD protein were collected and dialyzed against 25mM Tris, pH 8.7, 10mM NaCl, and then concentrated in a Speed-Vac concentrator to 1-2 ml. The protein sample was applied to a Superdex 75 column and eluted with 25mM Tris, pH 8.7, 150 mM NaCl. The 80 KD protein eluted as a homogenous protein.

C. 71 KD Extracellular Product

1. A 40-95% ammonium sulfate precipitate was obtained as discussed above with the exception that the 71 KD product was cultured in 7H9 broth at pH 7.4 and at 0% CO_2 and heat-shocked at 42°C for 3h once per week. The precipitate was dialyzed against Initial Buffer (20 mM Hepes, 2 mM MgAc, 25 mM KCl, 10 mM $(NH4)_2SO_4$, 0.8 mM DL-Dithiothreitol, pH 7.0).
2. The resolubilized precipitate was applied to an ATP Agarose column equilibrated with Initial Buffer. Effluent was collected and reapplied to the ATP Agarose column. The 71 KD protein bound to the column.
3. Subsequently the ATP Agarose column was washed, first with Initial Buffer, then 1 M KCl, then Initial Buffer.

4. Homogeneous 71 KD protein was eluted from the column with 10 mM ATP and dialyzed against phosphate buffer.

D. **58 KD Extracellular Product**

1. A 25-50% ammonium sulfate precipitate was obtained as discussed above.
2. The resolubilized precipitate was dialyzed and applied to a DEAE-Sepharose CL-6B or QAE-Sepharose column and eluted with NaCl. Collected fractions containing the 58 KD Protein eluted at approximately 400 mM NaCl.
3. Collected fractions were then applied to a Sepharose CL-6B size fractionation column. The protein eluted at approximately 670-700,000 Daltons.
4. The eluted protein was applied to a thiopropyl-sepharose column. The homogeneous 58 KD protein eluted at approximately 250-350 mM 2-mercapto-ethanol. The eluted protein was monitored using SDS-PAGE and exhibited the single band shown in Fig. 1A, col. 2.

E. **45 KD Extracellular Product**

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.
   b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.
2. a. A DEAE CL-6B column (Pharmacia) was charged with 2.5 mM Tris, pH 8.7 containing 1 M NaCl and equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.
   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to column. The column was then washed overnight with the same buffer.

c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 45 KD protein eluted at approximately 40 mM NaCl.

3. a. A Q-Sepharose HP (Pharmacia) column was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and re-equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to column with subsequent washing using the same buffer.

c. The column was eluted with 10-150 mM NaCl in 25 mM Tris, pH 8.7.

4. a. Fractions containing the 45 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentration to 1 ml in a Speed Vac concentrator.

b. Concentrate was Applied to Superdex 75 column equilibrated with 25 mM Tris 150 mM NaCl, pH 8.7. The product eluted as a homogeneous protein. The eluted protein was monitored using SDS-PAGE and resulted in the single band shown in Fig. 1B, col. 2.

F. 32 KD Extracellular Product (A)

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.

b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.

2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight with same buffer.

c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 32 KD protein eluted at approximately 70 mM NaCl.

3\. a. Fractions containing the 32 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

b. The concentrate was then Applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with this buffer. The 32 KD product eluted as homogeneous protein.

4\. a. A Q-Sepharose HP column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl, and re-equilibrated with 25 mM Tris, 10mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing in the same buffer.

c. The column was eluted with a 100-300 mM NaCl gradient. Labeled 32A, the homogeneous protein elutes at approximately 120 mM NaCl and is shown as a single band in Fig. 1B, col. 4.

G. 32 KD Extracellular Product (B)

1\. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.

b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.

2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight with same buffer.

   c. A preliminary salt gradient of 10 mM to 200 mM NaCl in 25 mM Tris, pH 8.7 was run, eluting various proteins. Following column equilibration, a second salt gradient (200 to 300 mM NaCl) was run. The 32 KD protein eluted at approximately 225 mM NaCl.

3. a. A Q-Sepharose HP column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl, and re-equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing in the same buffer.

   c. The column was eluted with a 200-300 mM NaCl gradient in the same buffer.

4. a. Fractions containing the 32 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

   b. The concentrate was then applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with the same buffer. The 32 KD product, labeled 32B to distinguish it from the protein of 32 KD separated using protocol H, eluted as homogeneous protein and is shown as a single band on Fig. 1B, col. 3.

H. 30 KD Extracellular Product

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.
   b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.
2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.
   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight with same buffer.
   c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 30 KD protein eluted at approximately 140 mM NaCl.
3. a. Fractions containing the 30 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.
   b. The concentrate was then Applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with this buffer. The 30 KD product eluted as homogeneous protein and is shown as a single band on Fig. 1B, col. 5.

I. 24 KD Extracellular Product

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.
   b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.
2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl

and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight with same buffer.

c. A preliminary salt gradient of 10 mM to 200 mM NaCl in 25 mM Tris, pH 8.7 was run, eluting various proteins. Following column equilibration a second salt gradient (200 to 300 mM NaCl) was run. The 24 KD elutes at approximately 250 mM NaCl.

3. a. A Q-Sepharose HP column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl, and re-equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing in the same buffer.

c. The column was eluted with a 200-300 mM NaCl gradient in the same buffer.

4. a. Fractions containing the 24 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

b. The concentrate was then applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with the same buffer. The 24 KD product eluted as homogeneous protein and is shown as a single band on Fig. 1B, col 7.

J. 23.5 KD Extracellular Product

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.

b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.

2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column prior to subsequent washing overnight with same buffer.

c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 23.5 KD protein eluted at approximately 80 mM NaCl.

3. a. A Q-Sepharose HP column was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl, and re-equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing in the same buffer.

c. The column was eluted with 100-300 mM NaCl in 25 mM Tris, pH 8.7.

d. Steps 3a to 3c were repeated.

4. a. Fractions containing 23.5 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

b. The concentrate was then applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with the same buffer. The 23.5 KD product eluted as homogeneous protein. The eluted protein was monitored using SDS-PAGE and resulted in the single band shown in Fig. 1B, col 6.

K. 23 KD Extracellular Product

1. a. Ammonium sulfate cuts of 0-25% (1h at 0°C) and 25-60% (overnight at 0°C) were discarded.
   b. A 60-95% ammonium sulfate cut was retained.
2. a. A DEAE CL-6B column (Pharmacia) was charged with 50 mM Bis-Tris pH 7.0 containing 1 M NaCl and equilibrated with 50 mM Bis-Tris, 100 mM NaCl, pH 7.0.
   b. The protein sample was dialyzed against 50 mM Bis-Tris, pH 7.0, 100 mM NaCl buffer and applied to the column before washing the column overnight with the same buffer.
   c. The column was eluted with a 100 to 300 mM NaCl linear gradient in 50 mM Bis-Tris pH 7.0.
   d. Fractions were collected containing the 23 KD protein which eluted at approximately 100-150 mM NaCl.
3. a. The protein fractions were dialyzed against 25 mM Tris, pH 8.7, 10 mM NaCl and concentrated to 1-2 ml on a Savant Speed Vac Concentrator.
   b. The concentrate was applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7. The product elutes as a homogeneous protein as is shown in Fig. 1B col. 8.

L. 16 KD Extracellular Product

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.
   b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.

2. a. A DEAE CL-6B column (Pharmacia) was charged with 2.5 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight in the same buffer.

   c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 16 KD protein eluted at approximately 50 mM NaCl.

3. a. Fractions containing 16 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

   b. The concentrate was then applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with the same buffer. A 16 KD product eluted as homogeneous protein. The eluted protein was monitored using SDS-PAGE and resulted in the single band shown in Fig. 1B, col. 9.

M. 14 KD Extracellular Product

1. a. A 0-25% ammonium sulfate cut (1 hour at 0°C) was discarded.

   b. The 25-60% ammonium sulfate cut (overnight at 0°C) was retained.

2. a. A DEAE CL-6B column (Pharmacia) was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl and then equilibrated with 25 mM Tris, 10 mM NaCl, pH 8.7.

   b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing overnight in the same buffer.

c. The column was eluted with a salt gradient (10 mM to 200 mM) in 25 mM Tris, pH 8.7 buffer. The 14 KD protein eluted at approximately 60 mM NaCl.

3. a. A Q-Sepharose HP column was charged with 25 mM Tris, pH 8.7 containing 1 M NaCl, and re-equilibrated with 25 mM NaCl, pH 8.7.

b. The protein sample was dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7 and applied to the column with subsequent washing in the same buffer.

c. The column was eluted with 10-150 mM NaCl in 25 mM Tris, pH 8.7.

d. Steps 3a through 3c were repeated.

4. a. Fractions containing 14 KD product were collected, pooled and dialyzed against 25 mM Tris, 10 mM NaCl, pH 8.7, before concentrating the protein sample to 1 ml in a Speed-Vac Concentrator.

b. The concentrate was then applied to a Superdex 75 column equilibrated with 25 mM Tris, 150 mM NaCl, pH 8.7 and eluted with this buffer. The 14 KD product eluted as homogeneous protein. The eluted protein was monitored using SDS-PAGE and resulted in the single band shown in Fig. 1C, col 2.

N. 12 KD Extracellular Products

1. A 0-10% ammonium sulfate precipitate was obtained (overnight at 4°C).
2. The resolubilized precipitate was applied to a S200 Sephacryl size fractionation column eluting the protein as a 12 KD molecule.
3. The protein fractions were applied to a DEAE-Sepharose CL-6B or QAE-Sepharose ion exchange

column and eluted with an NaCl gradient as previously described. Fractions containing two homogeneous proteins having molecular weights of approximately 12 KD eluted at approximately 300-350 mM NaCl and were collected. The proteins were labeled 12A and 12B and purified as a doublet shown in Fig. 1D, col. 2.

As illustrated in the SDS-PAGE profile of Fig. 1, the principal or majorly abundant extracellular proteins of *M. tuberculosis* were purified to homogeneity through the use of the protocols detailed in Examples 2A - 2N above. More particularly, Fig. 1 illustrates four exemplary 12.5% acrylamide gels developed using SDS-PAGE and labeled 1A, 1B, 1C, and 1D. The standard in lane 1 of gels 1A-1C has proteins with molecular weights of 66, 45, 36, 29, 24, 20, and 14 KD. In gel 1D the standard in lane 1 contains proteins with molecular weights of 68, 45, 31, 29, 20, and 14 KD. The lanes containing the respective purified extracellular products show essentially one band at the reported molecular weight of the individual protein. It should be noted that in gel 1 D the 12 KD protein runs as a doublet visible in lane 2. Sequence analysis shows that the lower 12 KD (or 12B KD band) is equivalent to the upper 12 KD (or 12A KD) band except that it lacks the first 3 N-terminal amino acids.

Further analysis of these individual exemplary majorly abundant extracellular products is provided in Fig. 2. More particularly Fig. 2 is a tabular compilation of N-terminal sequence data obtained from these purified extracellular products showing that the majority of the isolated products are indeed distinct. Proteins 32A, 32B and 30 all had the same 5 N-terminal amino acids therefore further sequencing was necessary to fully characterize and differentiate them. Fig. 3 shows the extended N-terminal amino acid sequences for these three purified secretory products. Different amino acids at positions 16, 31 and

36 demonstrate that these isolated proteins are distinct from one another despite their similarity in molecular weight.

In addition to proteins 30, 32A and 32B, extended N-terminal amino acid sequences of other majorly abundant extracellular products were determined to provide primary structural data and to uncover possible relationships between the proteins. Sequencing was performed on the extracellular products purified according to Example 2 using techniques well known in the art. Varying lengths of the N-terminal amino acid sequence, determined for each individual extracellular product, are shown below identified by the apparent molecular weight of the intact protein, and represented using standard one letter abbreviations for the naturally occurring amino acids. In keeping with established rules of notation, the N-terminal sequences are written left to right in the direction of the amino terminus to the carboxy terminus. Those positions where the identity of the determined amino acid is less than certain are underlined. Where the amino acid at a particular position is unknown or ambiguous, the position in the sequence is represented by a dash. Finally, where two amino acids are separated by a slash, the correct constituent has not been explicitly identified and either one may occupy the position in that sequence.

PROTEIN	**N-TERMINAL AMINO ACID SEQUENCE**						
	5	10	15	20	25	30	35
12 KD	FDTRL	MRLED	EMKEG	RYEVR	AELPG	VDPDK	DVDIM
	40	45					
	VRDGQ	LTIKA	ERT				
	5	10	15	20	25	30	
14 KD	ADPRL	QFTAT	TLSGA	PFDGA	S/NLQGK	PAVLW	

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                5        10        15        20        25        30
16 KD       AYPIT    GKLGS    ELTMT    DTVGQ    VVLGW    KVSDL

                35        40        45
            F/YKSTA  VIPGY    TV-EQ    QI

                5        10        15        20
23 KD       AETYL    PDLDW    DYGAL    EPHIS    GQ

                5        10
23.5 KD     APKTY    -EELK    GTD

                5        10        15        20        25        30        35
24 KD       APYEN    LMVPS    PSMGR    DIPVA    FLAGG    PHAVY    LLDAF

               40        45        50        55        60
            NAGPD    VSNWV    TAGNA    MMTLA    -KGIC/S

                5        10        15        20        25        30        35
30 KD       FSRPG    LPVEY    LQVPS    PSMGR    DIKVQ    FQSGG    NNSPA

               40
            VYLLD

                5        10        15        20        25        30        35
32A KD      FSRPG    LPVEY    LQVPS    PSMGR    DIKVQ    FQSGG    ANSP-

               40
            LYLLD

                5        10        15        20
32B KD      FSRPG    LPVEY    LQVPS    A-MGR    DI
```

	5	10	15	20	25	30
45 KD	DPEPA	PPVPD	DAASP	PDDAA	APPAP	ADPP-

	5	10	15	20
58 KD	TEKTP	DDVFK	LAKDE	KVLYL

	5	
71 KD	ARAVG	I

	5	
80 KD	TDRVS	VGN

	5	10	15	20	
110 KD	NSKSV	NSFGA	HDTLK	V-ERK	RQ

This sequence data, combined with physical properties ascertained using SDS-PAGE, allow these representative majorly abundant extracellular products of the present invention to be characterized and distinguished. The analysis described indicates that these proteins constitute the majority of the extracellular products of *M. tuberculosis*, with the 71 KD, 30 KD, 32A KD, 23 KD and 16 KD products comprising approximately 60% by weight of the total available extracellular product. It is further estimated that the 30 KD protein may constitute up to 25% by weight of the total products released by *M. tuberculosis*. Thus, individual exemplary majorly abundant extracellular products of *M. tuberculosis* useful in the practice of the present invention may range anywhere from approximately 0.5% up to approximately 25% of the total weight of the extracellular products.

As previously discussed, following the inability of traditional Western blot analysis to consistently identify the most immunogenically specific extracellular products, the present inventor decided to analyze the immunogenicity of the majorly abundant extracellular products based upon their abundance and consequent ease of identification and isolation. Surprisingly, it was found that these majorly abundant extracellular products induce unexpectedly effective immune responses leading this inventor to conclude that they may function as vaccines. This surprising discovery led to the development of the non-limiting functional theory of this invention discussed above.

To demonstrate the efficacy of the present invention, additional experiments were conducted using individual majorly abundant extracellular products and combinations thereof at various exemplary dosages to induce protective immunity in art accepted laboratory models. More specifically, purified individual majorly abundant extracellular products were used to induce protective immunity in guinea pigs which were then challenged with *M. tuberculosis*. Upon showing that these proteins were capable of inducing protective immunity, combinations of five purified majorly abundant extracellular products was similarly tested using differing routes of administration. In particular the 30 KD abundant extracellular product was used to induce protective immunity in the accepted animal model as was the purified form of the 71 KD extracellular product. As with the individual exemplary majorly abundant extracellular products the combination vaccines of five majorly abundant extracellular products conferred protection against challenge with lethal doses of *M. tuberculosis* as well. Results of the various studies of these exemplary vaccines of the present invention follow.

Specific pathogen-free male Hartley strain guinea pigs (Charles River Breeding Laboratories, North Wilmington, Massachusetts) were used in all experiments involving immunogenic or aerosol challenges with *M.*

tuberculosis. The animals were housed two or three to a stainless steel cage and allowed free access to standard guinea pig chow and water. After arrival at the animal facility, the guinea pigs were observed for at least one week prior to the start of each experiment to ensure that they were healthy.

Initial experiments were conducted using individual majorly abundant extracellular products believed to comprise between 3% to 25% of the total extracellular proteins normally present. These experiments demonstrate that majorly abundant extracellular products elicit an effective immune response. More particularly, isolated 30 KD and 71 KD extracellular products were shown to be individually capable of generating a cell-mediated immune response that protected guinea pigs upon exposure to lethal doses of *M. tuberculosis* as follows.

Example 3

Purified 30 KD Protein Skin Testing for Cell-Mediated Immunity of 30 KD Immunized Guinea Pigs

To illustrate that a measurable immune response can be induced by purified forms of abundant extracellular products, a cutaneous hypersensitivity assay was performed. Guinea pigs were immunized with the exemplary majorly abundant *M. tuberculosis* 30 KD secretory product purified according to Example 2 and believed to comprise approximately 25% of the total extracellular product of *M. tuberculosis*. In three independent experiments, guinea pigs were immunized three times three weeks apart with 100 μg of substantially purified 30 KD protein in SAF adjuvant. Control animals were similarly injected with buffer in SAF. Three weeks after the last immunization the guinea pigs were challenged with the exemplary 30 KD protein in a cutaneous hypersensitivity assay.

Guinea pigs were shaved over the back and injections of 0.1, 1 and 10 μg of 30 KD protein were administered intradermally with resulting erythema (redness of the skin) and induration measured after 24 hours as shown in Table A below. Data are reported in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods. ND indicates that this particular aspect of the invention was not done.

Table A

Erythema (mm) to 30 KD (Mean ± SE)

Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Expt. 1				
Immunized	6	1.2 ± 0.5	3.9 ± 0.8	6.9 ± 1.0
Controls	5	ND	ND	3.0 ± 0.9
Expt. 2				
Immunized	6	0.5 ± 0.5	5.4 ± 0.7	8.1 ± 0.6
Controls	3	0 ± 0	2.5 ± 0	1.7 ± 0.8
Expt. 3				
Immunized	6	ND	1.7 ± 1.1	6.2 ± 0.3
Controls	3	ND	ND	2.0 ± 0.0

Induration (mm) to 30 KD (Mean ± SE)

Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Expt. 1				
Immunized	6	0 ± 0	3.3 ± 0.3	5.6 ± 0.9
Controls	5	ND	ND	1.6 ± 1.0
Expt. 2				
Immunized	6	0 ± 0	3.8 ± 0.7	4.9 ± 1.2
Controls	3	0 ± 0	0.8 ± 0.8	1.7 ± 0.8
Expt. 3				
Immunized	6	ND	1.1 ± 1.1	4.7 ± 0.4
Controls	3	ND	0 ± 0	0 ± 0

As shown in Table A, guinea pigs immunized with the exemplary 30 KD secretory product exhibited a strong cell-mediated immune response as evidenced by marked erythema and induration. In contrast, the control animals exhibited minimal response.

To confirm the immunoreactivity of the 30 KD secretory product and show its applicability to infectious tuberculosis, non-immunized guinea pigs were infected with *M. tuberculosis* and challenged with this protein as follows.

Example 4

Purified 30 KD Protein Testing for Cell-Mediated Immune Responses of Guinea Pigs Infected With *M. tuberculosis*

To obtain bacteria for use in experiments requiring the infection of guinea pigs, *M. tuberculosis* was first cultured on 7H11 agar and passaged once through a guinea pig lung to insure that they were virulent. For this purpose, guinea pigs were challenged by aerosol with a 10 ml suspension of bacteria in 7H9 broth containing approximately 5×10^4 bacteria/ml. After the guinea pigs became ill, the animals were sacrificed and the lungs, containing prominent *M. tuberculosis* lesions, were removed. Each lung was ground up and cultured on 7H11 agar for 7 days to 10 days. The bacteria were scraped from the plates, diluted in 7H9 broth containing 10% glycerol, sonicated in a water bath to obtain a single cell suspension, and frozen slowly at -70°C at a concentration of approximately 2×10^7 viable bacteria/ml. Viability of the frozen cells was measured by thawing the bacterial suspension and culturing serial dilutions of the suspension on 7H11 agar. Just before a challenge, a vial of bacterial cells was thawed and diluted to the desired concentration in 7H9 broth.

The guinea pigs were exposed to aerosols of the viable *M. tuberculosis* in a specially designed lucite aerosol chamber. The aerosol chamber measured 14 by 13 by 24 in. and contained two 6 inch diameter portals on opposite sides for introducing or removing guinea pigs. The aerosol inlet was located at the center of the chamber ceiling. A vacuum pump (Gast Mfg. Co., Benton Harbor, Michigan) delivered air at 30 lb/in^2 to a nebulizer-venturi unit (Mes Inc., Burbank, California), and an aerosol was generated from a 10-ml suspension of bacilli. A 0.2 μm breathing circuit filter unit (Pall Biomedical Inc., Fajardo, Puerto Rico) was located at one end of the chamber to equilibrate the pressure inside and outside of the assembly. Due to safety considerations, the aerosol challenges were conducted with the chamber placed completely within a laminar flow hood.

The animals were exposed to pathogenic aerosol for 30 minutes during which time the suspension of bacilli in the nebulizer was completely exhausted. Each aerosol was generated from the 10 ml suspension containing approximately 5.0 x 10^4 bacterial particles per ml. Previous studies have shown that guinea pig exposure to this concentration of bacteria consistently produces infections in non-protected animals. Following aerosol infection, the guinea pigs were housed in stainless steel cages contained within a laminar flow biohazard safety enclosure (Airo Clean Engineering Inc., Edgemont, Pennsylvania) and observed for signs of illness. The animals were allowed free access to standard guinea pig chow and water throughout the experiment.

In this experiment, the infected guinea pigs were sacrificed and splenic lymphocyte proliferation was measured in response to various concentrations of the 30 KD protein. More specifically, splenic lymphocytes were obtained and purified as described by Brieman and Horwitz (*J. Exp. Med.* 164:799-811) which is incorporated herein by reference. The lymphocytes were adjusted to a final concentration of 10^7/ml in RPMI 1640 (GIBCO Laboratories, Grand Island, New York) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (GIBCO) and incubated with various concentrations of purified 30 KD secretory product in a total volume of 100 μl in microtest wells (96-well round-bottom tissue culture plate; Falcon Labware, Oxnard, California) for 2 days at

37°C in 5% CO_2-95% air and 100% humidity. Noninfected animals were used as negative controls. At the end of the incubation period, 0.25 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) was added to each well and the cells were further incubated for 2 hours at 37°C in 5% CO_2-95% air at 100% humidity. A multisample automated cell harvester (Skatron Inc., Sterling, Virginia) was used to wash each well, and the effluent was passed through a filtermat (Skatron). Filtermat sections representing separate microtest wells were placed in scintillation vials, and 2 ml of Ecoscint H liquid scintillation cocktail (National Diagnostics, Manville, New Jersey) was added. Beta particle emission was measured in a beta scintillation counter (Beckman Instruments Inc., Fullerton, California).

Tissue samples from the infected and noninfected guinea pigs were assayed against 1 and 10 μg/ml of isolated 30 KD secretory protein. Samples were then monitored for their ability to incorporate [^{3}H]thymidine. The results of these assays were tabulated and presented in Table B below.

Data are reported as a stimulation index which, for the purposes of this disclosure, is defined as:
mean [^{3}H]thymidine incorporation of lymphocytes incubated with antigen / mean [^{3}H]thymidine incorporation of lymphocytes incubated without antigen.

Table B

Guinea Pig Status	n	Stimulation Indices to 30 KD (Mean ± SE) 1.0 μg/ml	10.0 μg/ml
Infected	6	2.2 ± 0.2	9.7 ± 4.6
Controls	6	1.5 ± 0.3	2.0 ± 0.8

As shown in Table B, the cells of the infected animals exhibited a strong response to the exemplary 30 KD protein as manifested by dose dependant splenic lymphocyte proliferation in response to exposure to this majorly abundant secretory product. Conversely, the uninfected control animals showed little lymphocyte proliferation. Accordingly, the 30 KD secretory product clearly induces a cell-mediated immune response in mammals infected with *M. tuberculosis*.

To illustrate the protective aspects of the vaccines of the present invention, guinea pigs were immunized with purified 30 KD protein and exposed to *M. tuberculosis* as follows.

Example 5

Challenge of 30 KD Immunized Guinea Pig
With Aerosolized *M. tuberculosis*

As before, the animals were immunized three times at three week intervals with 100μg of the exemplary 30 KD secretory protein in SAF. Control guinea pigs were immunized with 120μg of bulk EP in SAF or sham-immunized with buffer in the same adjuvant. Three weeks after the last immunization, the animals were challenged with aerosolized *M. tuberculosis* as described in Example 4. The survival rates for the three groups of animals were monitored and are graphically presented in Fig. 4. Absolute mortality was determined 14 weeks after challenge as presented in Table C below.

Table C

Status of Guinea Pigs	Survivors/ Challenged	Percent Survival
30 KD Immunized	4/6	67%
EP Immunized	3/6	50%
Sham Immunized	1/6	17%

As shown in Fig. 4 guinea pigs immunized three times with the exemplary 30 KD protein were protected against death. Approximately 67% of the guinea pigs immunized with the 30 KD protein survived whereas only 17% of the control sham-immunized guinea pigs survived.

Weight retention of the immunized animals was also monitored (data not shown) and further illustrates the prophylactic capacity of vaccines incorporating majorly abundant extracellular products produced by pathogenic bacteria as taught by the present invention. While the immunized animals appeared to maintain their weight, the high mortality rate of the sham-immunized animals precluded the graphical comparison between the immunized animals and the control animals.

Following conclusion of the weight monitoring study, the surviving animals were sacrificed and the right lung and spleen of each animal was assayed for viable *M. tuberculosis*. The animals were soaked in 2% amphyl solution (National Laboratories, Montvale, New Jersey), and the lungs and spleen were removed aseptically. The number of macroscopic primary surface lesions in the lungs were enumerated by visual inspection. Colony forming units (CFU) of *M. tuberculosis* in the right lung and spleen were determined by homogenizing each organ in 10 ml of 7H9 with a mortar and pestle and 90-mesh Norton Alundum (Fisher), serially diluting the tissue homogenate in 7H9, and culturing the dilutions on duplicate plates of 7H11 agar by using drops of 0.1 ml/drop. All plates were kept in modular incubator chambers and incubated 12 to 14 days at 37°C in 5% CO_2, 95% air at 100% humidity. The assay was conducted using this protocol and the results of the counts are presented in Table D below in terms of mean colony forming units (CFU) ± standard error (SE).

Table D

Guinea Pig Status	n	Mean CFU ± SE Right Lung	Spleen
30 KD Immunized	4	$3.4 \pm 1.7 \times 10^7$	$7.7 \pm 3.9 \times 10^6$
Sham-immunized	1	1.8×10^8	8.5×10^7
Log-Difference		0.73	1.04

As shown in Table D, immunization with the exemplary 30 KD secretory protein limited the growth of *M. tuberculosis* in the lung and the spleen. Although only data from the one surviving sham-immunized animal was available for comparative purposes, the four surviving 30 KD immunized animals had 0.7 log fewer CFU in their lungs and 1 log fewer CFU in their spleen than the surviving sham-immunized animal. Based on previous demonstrations of a high correlation between CFU counts and mortality, the surviving animal likely had fewer CFU in the lungs and spleen than the animals who died before a CFU analysis could be performed. Again this reduction of CFU in the lungs and spleens of the immunized animals conclusively demonstrates the scope and operability of the present invention.

The immunoprotective potential of another majorly abundant extracellular product from *M. tuberculosis,* the 71 KD extracellular product, was tested in its isolated form to demonstrate its immunoprotective capacity.

Example 6

Purified 71 KD Protein Skin Test of Guinea Pigs Immunized with a Bulk Preparation of EP

To demonstrate the potential of 71 KD protein to provoke an effective immune response in animals, this isolated majorly abundant extracellular product was used to skin test guinea pigs immunized with a bulk preparation of *M. tuberculosis* extracellular proteins (EP) in a cutaneous hypersensitivity assay. As discussed above, bulk EP will impart acquired immunity against infection by *M. tubercu-*

losis but to a lesser extent than the vaccines of the present invention.

Guinea pigs were immunized on two occasions spaced three weeks apart, with 120 μg of a bulk preparation of EP prepared as detailed in Example 1. The vaccination was prepared in incomplete Freunds adjuvant with sham-immunized animals receiving buffer in place of EP. Three weeks after the last vaccination the guinea pigs from each group were shaved over the back and skin tested with an intradermal injection of 0.1, 1.0 and 10 μg of 71 KD protein. 10.0 μg of buffer was used as a control and all injections were performed using a total volume of 0.1 ml. The diameters of erythema and induration were measured after 24 hours with the results as shown in Table E below. Data are reported in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods.

Table E

		Erythema (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Immunized	4	6.5 ± 0.7	11.9 ± 1.4	18.9 ± 2.2
Controls	3	2.5 ± 1.4	5.0 ± 2.9	11.8 ± 2.1

		Induration (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Immunized	4	3.6 ± 1.1	6.8 ± 1.1	11.6 ± 0.8
Controls	3	0.7 ± 0.7	3.7 ± 0.9	7.8 ± 1.0

The responses of the immunized animals were almost twice the response of the guinea pigs challenged with buffer alone and were comparable to those challenged with bulk EP identical to that used to immunize the animals (data not shown).

To further confirm that the purified exemplary 71 KD majorly abundant extracellular product elicits cell-mediated immune responses, the bulk EP immunized guinea pigs were sacrificed and splenic lymphocyte proliferation was measured in response to various concentrations of the 71 KD protein. Nonimmunized animals were used as controls. Following the protocol of Example 4, the lymphocytes were incubated with and without 71 KD protein for 2 days and then assayed for their capacity to incorporate [^{3}H]thymidine.

Data is reported in terms of stimulation indices calculated as in Example 4. The results of this 71 KD challenge are shown in Table F below.

Table F

Guinea Pig Status	n	Stimulation Indices to 71 KD (Mean ± SE) 0.01 μg/ml	0.1 μg/ml	1.0 μg/ml
Immunized	4	1.5 ± 0.1	2.3 ± 0.5	8.1 ± 2.2
Controls	2	1.7 ± 0.6	1.6 ± 0.4	2.5 ± 0.6

Guinea Pig Status	n	Stimulation Indices to EP (Mean ± SE) 0.01 μg/ml	0.1 μg/ml	1.0 μg/ml
Immunized	4	1.5 ± 0.1	2.2 ± 0.3	5.3 ± 1.4
Controls	2	1.4 ± 0.2	1.5 ± 0.2	1.2 ± 0.1

As shown in Table F, stimulation indices for the lymphocyte proliferation assay were comparable to the results obtained in the cutaneous hypersensitivity assay. Both the 71 KD and bulk EP tested samples showed responses between two and three times higher than those obtained with the controls indicating that isolated exemplary 71 KD majorly abundant extracellular product is capable of provoking a cell-mediated immune response in animals immunized with *M. tuberculosis* extracts. However, it should again be emphasized that the purified majorly abundant or principal extracellular product is free of the problems associated with prior art or bulk compositions and is more readily adaptable to synthetic and commercial production making the vaccines of the present invention superior to the prior art.

More particularly the bulk preparation cannot be manufactured easily on a large scale through modern biomolecular techniques. Any commercial production of these unrefined bulk preparations containing all extracellular products would involve culturing vast amounts of the target pathogen or a closely related species and harvesting the resultant supernatant fluid. Such production methodology is highly susceptible to contamination by the target pathogen, toxic byproducts or other parasitic agents. Further, the large number of immunogenic determinants in such a preparation is far more likely to provoke a toxic immune reaction in a susceptible segment of the immunized population. Using these unrefined bulk preparations also negates the use of the most popular skin tests currently used for tuberculosis screening and control.

In direct contrast, the vaccines of the present invention can be mass-produced in relative safety using high yield transformed hosts. Similarly, the vaccines of the present invention can be produced in identical, easy to standardize batches as opposed to the wider variable production of bulk extracellular products. Moreover, as the number of immunogenic determinants presented tc the host immune system is relatively small, toxic reactions and the chance of invalidating popular screening tests are greatly reduced.

Example 7

Purified 71 KD Protein Skin Test of 71 KD Immunized Guinea Pigs

Following demonstration that the isolated exemplary 71 KD majorly abundant extracellular product generates a cell-mediated immune response in bulk EP immunized animals, it was shown that the purified form of this majorly abundant product was able to induce a cell-mediated immune response in animals immunized with 71 KD.

Guinea pigs were twice vaccinated with 100 μg of purified 71 KD protein in SAF three weeks apart. Control animals were sham-immunized with buffer in SAF on the same schedule. Three weeks after the last immunization both sets of animals were intradermally challenged with 1 and 10 μg of isolated 71 KD protein. The resulting erythema and indurations were measured after 24 hours with the results shown in Table G below.

Table G

		Erythema (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0 μg	1.0 μg	10.0 μg
Immunized	3	0 ± 0	6.5 ± 1.5	15.0 ± 1.5
Controls	3	0 ± 0	2.7 ± 1.3	6.7 ± 1.3

		Induration (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0 μg	1.0 μg	10.0 μg
Immunized	3	0 ± 0	3.0 ± 1.0	9.3 ± 0.3
Controls	3	0 ± 0	0 ± 0	1.3 ± 1.3

The extent of induration and erythema was much greater in the immunized animals than in the non-immunized control animals demonstrating that a strong cell-mediated immune response to 71 KD protein had been initiated by the vaccination protocol of the present invention.

To further confirm the capacity of this abundant extracellular product to induce an effective immune response on its own in accordance with the teachings of the present invention, lymphocyte proliferation assays were performed. Animals immunized as in Table G were sacrificed and splenic lymphocyte proliferative assays were run using the protocol established in Example 4. The tissue samples from the 71 KD immunized guinea pigs and those from the control guinea pigs were challenged with 0.1, 1 and 10 μg/ml of isolated 71 KD protein and monitored for their ability to incorporate [^{3}H]thymidine. Stimulation indices were calculated as previously described. The results of these assays are presented in Table H below.

Table H

Guinea Pig Status	n	Stimulation Indices to 71 KD (Mean ± SE)		
		0.1 μg/ml	1.0 μg/ml	10.0 μg/ml
Immunized	3	4.0 ± 1.3	5.6 ± 2.5	12.2 ± 5.1
Controls	3	1.3 ± 0.3	1.3 ± 0.3	3.2 ± 1.5

As with the cutaneous hypersensitivity assay, the 71 KD immunized animals showed a much higher response to purified 71 KD than did the sham-immunized controls. Though expected of a foreign protein, such results clearly show that a majorly abundant extracellular product has the capacity to induce an cell-mediated immune response.

After establishing that an isolated majorly abundant extracellular protein will induce an effective cell-mediated immune response, further experiments were conducted to confirm that any such response is cross-reactive against tubercle bacilli as follows.

Example 8

Purified 71 KD Protein Challenge of Guinea Pigs Infected With *M. tuberculosis*

Non-immunized guinea pigs were infected with aerosolized *M. tuberculosis* as reported in Example 4. Purified protein derivative (PPD-CT68; Connaught Laboratories Ltd.) was employed as the positive control to ensure that the infected animals were demonstrating a cell-mediated immune response indicative of *M. tuberculosis*. Widely used in the Mantoux test for tuberculosis exposure, PPD is generally prepared by ammonium sulfate fractionation and comprises a mixture of small proteins having an average molecular weight of approximately 10 KD. Immune responses to PPD are substantially analogous to those provoked by the bulk EP fractions isolated in Example 1.

Three weeks after infection the guinea pigs were challenged intradermally with 0.1, 1 and 10 μg of the exemplary purified majorly abundant 71 KD extracellular protein. Uninfected animals used as controls were similarly challenged with the isolated protein. The extent of erythema and induration were measured 24 hours later with the results reported in Table I below.

Table I

		Erythema (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Infected	7	9.5 ± 1.7	13.4 ± 1.3	19.7 ± 1.3
Controls	6	2.3 ± 2.3	3.5 ± 2.2	7.8 ± 1.9

		Induration (mm) to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg	1.0 μg	10.0 μg
Infected	7	5.3 ± 1.8	8.7 ± 1.6	13.4 ± 1.1
Controls	6	0 ± 0	0.8 ± 0.8	0 ± 0

As shown in Table I, strong immune responses are present in the infected animals challenged with the exemplary purified majorly abundant extracellular protein of the present invention. These responses are on the order of three to four times greater for erythema and more than 10 times greater for induration than those of the uninfected animals, confirming that the prominent 71 KD extracellular protein induces a strong cell-mediated immune response in *M. tuberculosis*-infected animals.

To further corroborate these results the infected animals and uninfected animals were sacrificed and subjected to a lymphocyte proliferative assay according to the protocol of Example 4. The tissue samples from both sets of guinea pigs were assayed against 0.1, 1 and 10 μg/ml of isolated 71 KD protein and PPD. The samples were then monitored for their ability to incorporate [^{3}H]thymidine as previously described with the results of these assays presented in Table J below.

Table J

		Stimulation Indices to 71 KD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg/ml	1.0 μg/ml	10.0μg/ml
Infected	3	2.4 ± 0.5	6.2 ± 1.8	29.1 ± 16.2
Controls	3	1.1 ± 0.1	2.6 ± 0.8	18.2 ± 6.1

		Stimulation Indices to PPD (Mean ± SE)		
Guinea Pig Status	n	0.1 μg/ml	1.0 μg/ml	10.0μg/ml
Infected	3	1.0 ± 0.1	4.0 ± 1.5	11.4 ± 3.4
Controls	3	0.9 ± 0.2	0.9 ± 0.03	1.5 ± 0.3

As with the results of the cutaneous sensitivity assay, Table J shows that the stimulation indices were much higher for the infected tissue than for the uninfected samples. More specifically, the mean peak stimulation index of infected animals was 2-fold higher to the exemplary 71 KD protein and 3-fold higher to PPD than it was to uninfected controls confirming that a strong cell-mediated immune response is induced in animals infected with *M. tuberculosis* by the exemplary majorly abundant extracellular protein vaccines of the present invention.

Following this demonstration of cross-reactivity between the exemplary purified 71 KD majorly abundant protein and *M. tuberculosis*, additional experiments were performed to demonstrate that an effective immune response could be stimulated by these exemplary purified samples of the majorly abundant extracellular products as disclosed by the present invention.

Example 9

Challenge of 71 KD Immunized Guinea Pigs With Aerosolized *M. tuberculosis*

To demonstrate the immunoprotective capacity of exemplary majorly abundant or principal extracellular protein vaccines, guinea pigs were immunized twice, 3 weeks apart, with 100 μg of the exemplary majorly abundant 71 KD protein purified according to Example 2. Control animals were immunized with 120 μg bulk EP from Example 1 or buffer. All animals were immunized using the adjuvant SAF. Three weeks after the last immunization, guinea pigs immunized with the exemplary 71 KD protein were skin-tested with 10 μg of the material to evaluate whether a cell-mediated immune response had developed. The control animals and 71 KD immunized guinea pigs were then infected with aerosolized *M. tuberculosis* as detailed in Example 4. Following infection the animals were monitored and weighed for six months.

The graph of Fig. 5 contrasts the weight loss experienced by the sham-immunized group to the relatively normal weight gain shown by the 71 KD and bulk EP immunized animals. Data are the mean weights ± SE for each group. Mortality curves for the same animals are shown in the graph of Fig. 6. The absolute mortality rates for the study are reported in Table K below.

Table K

Status of Guinea Pigs	Survivors/ Challenged	Percent Survival
71 KD Immunized	3/6	50%
EP Immunized	5/8	62.5%
Sham Immunized	0/6	0%

Both the weight loss curves and the mortality rates clearly show that the majorly abundant extracellular proteins of the present invention confer a prophylactic immune response. This is emphasized by the fact that 100% of the non-immunized animals died before the end of the monitoring period.

Example 10

Challenge of 71 KD Immunized Guinea Pigs With Aerosolized *M. tuberculosis*

A similar experiment was conducted to verify the results of the previous Example and show that the administration of an exemplary principal extracellular protein can confer a protective immune response in animals. In this experiment, guinea pigs were again immunized three times, 3 weeks apart, with 100μg of the 71 KD extracellular protein in SAF. Control guinea pigs were sham-immunized with buffer in SAF. Three weeks after the last immunization, the animals were challenged with aerosolized *M. tuberculosis* and weighed weekly for 13 weeks. Mean weights ± SE for each group of 6 guinea pigs were calculated and are graphically represented in Fig. 7. This curve shows that the sham-immunized animals lost a considerable amount of weight over the monitoring period while the immunized animals maintained a fairly consistent body weight. As loss of body mass or "consumption" is one of the classical side effects of tuberculosis, these results indicate that the growth and proliferation of tubercle bacilli in the immunized animals was inhibited by the exemplary vaccine of the present invention.

Protective immunity having been developed in guinea pigs through vaccination with an abundant extracellular product in an isolated form, experiments were run to demonstrate the inter-species immunoreactivity of the vaccines of the present invention and to further confirm the validity and applicability of the guinea pig model.

Example 11

Testing Cell-Mediated Immunity of PPD Positive Humans With Purified 71 KD Protein

To assess the cell-mediated component of a human immune response to the exemplary 71 KD majorly abundant protein, the proliferation of peripheral blood lymphocytes from PPD-positive and PPD-negative individuals to the protein were studied in the standard lymphocyte proliferation assay as reported in Example 4 above. A positive PPD, or tuberculin, response is well known in the art as being indicative of previous exposure to *M. tuberculosis*. The proliferative response and corresponding incorporation of [^{3}H]thymidine were measured at two and four days. Data for these studies is shown in Figs. 8A and 8B. Fig. 8A shows the response to various levels of 71 KD after two days while Fig. 8B shows the same responses at four days.

As illustrated in Figs. 8A and 8B, the mean peak stimulation index of PPD-positive individuals was twofold higher to the 71 KD protein and threefold higher to PPD than that of PPD negative individuals. Among PPD-positive individuals, there was a linear correlation between the peak stimulation indices to the exemplary 71 KD protein and to PPD demonstrating that a strong cell-mediated response is stimulated by the most prominent or majorly abundant extracellular products of *M. tuberculosis* in humans previously exposed to *M. tuberculosis*. This data corresponds to the reactivity profile seen in guinea pigs and confirms the applicability of the guinea pig model to other mammals subject to infection.

Thus, as with the previously discussed 30 KD exemplary protein, the development of a strong immune response to the majorly abundant 71 KD extracellular product demonstrates the broad scope of the present invention as evidenced by the fact that the 71 KD product is also effective at stimulating cell-mediated immunity in humans.

Again, it should be emphasized that the present invention is not limited to the extracellular products of *M. tuberculosis* or to the use of the exemplary 71 KD protein. Rather the teachings of the present invention are applicable to any majorly abundant extracellular product as demonstrated in the examples.

Additional studies were performed in order to ascertain whether combinations of majorly abundant extracellular products of *M. tuberculosis* would provide protective immunity as well. In general, these studies utilized guinea pigs which were immunized either intradermally or subcutaneously with various dosages of vaccines comprising combinations of 5 purified extracellular proteins of *M. tuberculosis* in SAF three times, 3 or 4 weeks apart.

The first protein combination used for the immunization procedure, labeled Combination I, was comprised of 71 KD, 32A KD, 30 KD, 23 KD, and 16 KD proteins purified according to the protocols described in Example 2. This combination is believed to comprise up to 60% of the total extracellular protein normally present in *M. tuberculosis* culture supernatants. These proteins selected for use in Combination I, are identified with an asterisk in Fig. 2. Combination I vaccine containing 100 μg, 20 μg, or 2 μg of each protein was administered intradermally with the adjuvant SAF. Combination I vaccine containing 20 μg of each protein was also administered subcutaneously in similar experiments. Negative control guinea pigs were sham-immunized with equivalent volumes of SAF and buffer on the same schedule while positive controls were immunized using

120 μg of the bulk extracellular protein preparation from Example 1 in SAF. All injection volumes were standardized using buffer.

Example 12

Response of Combination I Immunized Guinea Pigs to a Challenge With Combination I Vaccine

To determine if the animals had developed a measurable immune response following vaccination with the Combination I mixture of principal extracellular products, a cutaneous hypersensitivity assay was performed. Guinea pigs were shaved over the back and injected intradermally with 1.0 μg and 10.0 μg of the same combination of the five purified extracellular proteins. 10.0 μg of buffer was used as a control and all injections were performed using a total volume of 0.1 ml. The diameters of erythema and induration at skin tests sites were measured at 24 hours after injection.

The results of the measurements are presented in Table L below. Data are again reported in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods. ND indicates that this particular aspect of the experiment was not done.

Table L

Guinea Pig Status	n	PD	Erythema (mm) (Mean ± SE) 1.0 μg	Erythema (mm) (Mean ± SE) 10.0 μg
Immunized	6	0	11.4 ± 4.6	17.4 ± 2.6
Controls	6	0	ND	6.0 ± 0.5

	n	PD	Induration (mm) (Mean ± SE) 1.0 μg	Induration (mm) (Mean ± SE) 10.0 μg
Immunized	6	0	7.3 ± 0.8	11.6 ± 1.2
Controls	6	0	ND	4.2 ± 0.3

The data clearly demonstrate that a strong cell-mediated immune response to the Combination I extracellular proteins was generated by the vaccinated animals. The immunized guinea pigs show erythema and induration measurements almost three times greater than the control animals.

Example 13

<u>Immunoprotective Analysis of Combination I Vaccine Against Aerosolized *M. tuberculosis*</u>

Three weeks after the last immunization, the guinea pigs used for the preceding hypersensitivity assay were challenged with aerosolized *M. tuberculosis*, Erdman strain and weighed weekly for 10 weeks. This aerosol challenge was performed using the protocol of Example 4. Six animals immunized with 100 μg of the principal extracellular products of Combination I, along with equal sized groups of positive and negative controls, were challenged simultaneously with aerosolized *M. tuberculosis*. Positive controls were immunized three times with 120 μg EP in SAF.

Guinea pigs that died before the end of the observation period were autopsied and examined for evidence of gross tuberculosis lesions. Such lesions were found in all animals which expired during the study.

Differences between immunized and control animals in mean weight profiles after aerosol challenge were analyzed by repeated measures analysis of variance (ANOVA). Differences between immunized and control guinea pigs in survival after challenge were analyzed by the two-tailed Fisher exact test. Data are the mean weights ± standard error (SE) for each group of six guinea pigs.

Results of the weekly weight determinations following challenge are shown in Fig. 9. Compared with guinea pigs immunized with the combination of extracellular products, sham-immunized animals lost 15.9% of their total body weight. Weights of the positive controls were similar to those of animals immunized with the combination of five purified extracellular proteins. Body weights were normalized immediately before challenge. The difference between animals immunized with Combination I and sham-immunized controls was highly significant with $p < .0000001$ by repeated measures ANOVA.

Mortality was determined ten and one-half weeks after challenge. All three of the sham-immunized animals died within three days of each other between ten and ten and one-half weeks after challenge. The mortality results of the experiment are provided in Table M below.

Table M

Status of Guinea Pigs	Survivors/ Challenged	Percent Survival
Combination Immunized	6/6	100%
EP-Immunized	5/6	83%
Sham-Immunized	3/6	50%

Following the conclusion of the weight monitoring study, the surviving animals were sacrificed by hypercarbia and the right lung and spleen of each animal was assayed for viable *M. tuberculosis* using the protocol of Example 5. The results of the counts, including the 3 animals that died the last week of the experiment, are presented in Table N below in terms of mean colony forming units (CFU) ± standard error (SE).

Table N

Guinea Pig Status	n	Mean CFU ± SE: Right Lung	Mean CFU ± SE: Spleen
Sham-immunized	6	$8.9 \pm 5.4 \times 10^7$	$1.3 \pm 0.7 \times 10^7$
Immunized	6	$3.4 \pm 1.7 \times 10^6$	$1.8 \pm 0.6 \times 10^6$
EP-immunized	6	$1.7 \pm 0.7 \times 10^7$	$5.0 \pm 2.8 \times 10^6$

The log difference between the concentration of bacilli in the lung of the animals immunized with the combination of purified proteins and that of the sham-immunized animals was 1.4 while the log difference of bacilli in the spleen was 0.9. Parallelling this, on gross inspection at autopsy immunized animals had markedly decreased lung involvement with tuberculosis compared with sham-immunized controls. Positive control animals immunized with the bulk extracellular preparation (EP) of Example 1 showed 0.7 log more bacilli in the lung and .5 log more bacilli in the spleen than animals immunized with the Combination I mixture of purified extracellular proteins.

Example 14

Immunoprotection Analysis of Combination I Vaccine at Low Doses Through Intradermal and Subcutaneous Delivery

While Example 13 confirmed that Combination I proteins demonstrated immunoprotection in animals immunized intradermally with 100 μg of each protein (30 + 32A + 16 + 23 + 71) 3 times, 4 weeks apart, an alternative study was conducted to demonstrate the immunoprotective capacity of lower doses of Combination I proteins, specifically 20 μg or 2 μg of each protein. As in Example 13, guinea pigs (6 animals per group) were immunized with Combination I proteins (30 + 32A + 16 + 23 + 71) intradermally in SAF 4 times, 3 weeks apart. Animals received either 20 μg or each protein per immunization or 2 μg of each protein per immunization. Control animals were sham-immunized utilizing the previous protocol. Three weeks later, the animals were challenged with aerosolized *M. tuberculosis* and weights were measured weekly for 9 weeks. All immunized animals survived to the end of the experiment while one sham-immunized animal died before the end of the experiment. As the following results illustrate, doses 5 fold and even 50 fold lower than those of Example 13 protected immunized animals from aerosolized *M. tuberculosis* and that delivery by both the intradermal and subcutaneous route was effective.

Compared with guinea pigs immunized with 20 μg of each protein of Combination I, sham-immunized animals lost 12 % of their total body weight during the 9 weeks of the experiment (weights were normalized to just before challenge). Compared with guinea pigs immunized with 2 μg of each protein of Combination I, sham-immunized animals lost 11% of their normalized total body weight. Thus, guinea pigs immunized intradermally with low doses of Combination I proteins were protected against weight loss after aerosol challenge with *M. tuberculosis*.

Similarly, guinea pigs immunized intradermally with low doses of Combination I proteins also were protected against splenomegaly associated with dissemination of *M. tuberculosis* to the spleen. As shown in Table O, whereas animals immunized with 20 μg or 2 μg of each protein of Combination I had spleens weighing an average of 4.6 ± 1.2g and 4.0 ± 0.8g (Mean ± SE), respectively, sham-immunized animals had spleens weighing an average of 9.6 ± 1.8g (Table 1), or more than twice as much.

Table O

Status of Guinea Pigs	n	Spleen Weight (g) Mean ± SE
Sham-Immunized	5	9.6 ± 1.8
Immunized (20 μg)	6	4.6 ± 1.2
Immunized (2 μg)	6	4.0 ± 0.8

Guinea pigs immunized intradermally with low doses of Combination I proteins also had fewer CFU of *M. tuberculosis* in their spleens. As shown in Table P, when compared with sham-immunized animals, guinea pigs immunized with 20 μg or 2 μg of each protein of Combination I had an average of 0.6 and 0.4 log fewer CFU, respectively, in their spleens.

Table P

Guinea Pig Status	n	CFU in Spleen Mean ± SE	Log Difference
Sham-Immunized	5	$3.1 \pm 2.3 \times 10^6$	
Immunized (20 μg)	6	$8.1 \pm 2.4 \times 10^5$	-0.6
Immunized (2 μg)	6	$1.2 \pm 0.6 \times 10^6$	-0.4

Moreover, guinea pigs immunized subcutaneously with Combination I proteins were also protected against weight loss, splenomegaly, and growth of *M. tuberculosis* in the spleen. In the same experiment described in Example 14, guinea pigs were also immunized subcutaneously rather than intradermally with 20 μg of Combination I proteins, 4 times, 3 weeks apart. These animals were protected from challenge almost as much as the animals immunized intradermally with 20 μg of Combination I proteins.

Example 15

<u>Response of Combination I and Combination II Immunized Guinea Pigs to Challenge with Combination I and Combination II</u>

Additional studies were performed to ascertain whether other combinations of majorly abundant extracellular products of *M. tuberculosis* would provide protective immunity as well. One study utilized guinea pigs which were immunized with a vaccine comprising two combinations - Combination I (71, 32A, 30, 23, and 16) and Combination II (32A, 30, 24, 23, and 16). Combination II is believed to comprise up to 62% of the total extracellular protein normally present in *M. tuberculosis* supernatants. Animals (6 per group) were immunized four times with 100 μg of each protein in Combination I or II in SAF, 3 weeks apart. Negative control animals were sham-immunized with equivalent volumes of SAF and buffer on the same schedule.

As in Example 12, the animals were tested for cutaneous delayed-type hypersensitivity to determine if the animals developed a measurable immune response following vaccination. Animals immunized with Combination II had 16.8 ± 1.3mm (Mean ± SE) erythema and 12.8 ± 1.2mm induration in response to skin-testing with Combination II whereas sham-immunized animals had only 1.3 ± 0.8mm erythema and 0.3 ± 3mm induration in response to Combination II. Thus, animals immunized with Combination II had greater than 12 fold more erythema and greater than 40 fold more induration than controls. By way of comparison, animals immunized with Combination I had 21.3 ± 2.0mm erythema and 15.8 ± 0.1mm induration in response to skin-testing with Combination I, whereas sham-immunized animals had only 6.4 ± 0.8mm erythema and 2.6 ± 0.7mm induration in response to Combination I. Thus, animals immunized with Combination I had greater than 3 fold more erythema and greater than 6 fold more induration than controls. The difference from controls for Combination II proteins was even greater than that for Combination I proteins.

In the same experiment, animals immunized with a lower dose of Combination II proteins (20 μg of each protein vs. 100 μg) also developed strong cutaneous hypersensitivity to Combination II. They had 21.0 ± 2.0mm erythema and 15.3 ± 0.9mm induration in response to Combination II, whereas the sham-immunized animals had only 1.3 ± 0.8mm erythema and 0.3 ± 0.3mm induration, as noted above. Thus, animals immunized with a lower dose of Combination II proteins had greater than 16 fold erythema and greater than 50 fold more induration than controls, a difference that was even greater than for animals immunized with the higher dose of Combination II proteins.

Example 16

<u>Immunoprotective Analysis of Combination I and II Vaccine Against Aerosolized *M. tuberculosis*</u>

Three weeks after the last immunization, the guinea pigs used for the preceding hypersensitivity assay were challenged with aerosolized *M. tuberculosis*, Erdman strain as in Example 13 and weighed weekly for 7 weeks. As in

Example 13, 6 animals were in each group. During the first 7 weeks after challenge, sham-immunized animals lost an average of 19.5g. In contrast, animals immunized with Combination II (100 μg of each protein) gained 52.4 g and animals immunized with Combination II at a lower dose (20 μg of each protein) gained an average of 67.2g. By way of contrast, animals immunized with Combination I gained 68g. Thus, compared with guinea pigs immunized with Combination II (100 μg), sham-immunized animals lost 11% of their total body weight. Compared with guinea pigs immunized with Combination II at a lower dose (20 μg), sham-immunized animals lost 14% of their total body weight. Compared with animals immunized with Combination I, sham-immunized animals also lost 14% of their total body weight.

Example 17

Response of Guinea Pigs Immunized with Combinations III through XII to a Challenge with the Same Vaccine or Its Components

Additional experiments were performed to demonstrate the effectiveness of various combinations of *M. tuberculosis* majorly abundant extracellular products. In these studies, Hartley type guinea pigs were immunized intradermally with vaccines comprising combinations of 2 or more majorly abundant extracellular products purified as in Example 2. The purified extracellular products are identified using their apparent molecular weight as determined by SDS-PAGE. The guinea pigs were immunized with the following combinations of majorly abundant extracellular products.

Combination	Protein Constituents
III	30 + 32A + 32B + 16 + 23
IV	30 + 32A
V	30 + 32B
VI	30 + 16
VII	30 + 23
VIII	30 + 71
IX	30 + 23.5
X	30 + 12
XI	30 + 24
XII	30 + 58

Each combination vaccine included 100 μg of each listed protein. The combination vaccines were volumetrically adjusted and injected intradermally in the adjuvant SAF. As before the guinea pigs were immunized four times, three weeks apart.

A cutaneous hypersensitivity assay was performed to determine if the animals had developed a measurable immune response following vaccination with the Combinations III to XII. Groups of six guinea pigs were shaved over the back and injected intradermally with the same combination of purified extracellular products to which they were immunized. For this challenge 10 μg of each of the proteins in the combination were injected. All injections were performed using a total volume of 0.1 ml. Sham-immunized controls, which had been immunized with SAF only were also skin-tested with Combinations III to XII, again using 10 μg of each protein in the respective combination. The diameters of erythema and induration at skin tests sites were measured 24 hours after injection as described in Example 3.

The results of these measurements are presented in Table Q below. Data are again reported in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods.

Table Q

Vaccine Combination	Skin Test Combination	Diameter of Skin Reaction (mm)	
		Erythema	Induration
III	III	12.2 ± 2.0	6.8 ± 0.8
IV	IV	9.9 ± 0.5	6.3 ± 0.2
V	V	13.0 ± 1.1	8.1 ± 0.7
VI	VI	19.2 ± 1.2	12.4 ± 0.5
VII	VII	14.3 ± 1.0	8.7 ± 0.4
VIII	VIII	18.9 ± 1.1	12.6 ± 0.8
IX	IX	17.0 ± 0.9	12.1 ± 0.9
X	X	19.3 ± 1.4	13.6 ± 1.2
XI	XI	18.3 ± 1.2	12.4 ± 0.8
XII	XII	17.7 ± 0.9	14.0 ± 1.2
Sham	III	4.8 ± 0.9	2.0 ± 0.0
Sham	IV	4.3 ± 1.1	2.0 ± 0.0
Sham	V	5.0 ± 0.5	2.0 ± 0.0
Sham	VI	4.5 ± 0.3	2.0 ± 0.0
Sham	VII	4.5 ± 0.3	2.0 ± 0.0
Sham	VIII	3.3 ± 0.3	2.3 ± 0.3
Sham	IX	3.7 ± 0.3	2.0 ± 0.0
Sham	X	3.7 ± 0.4	2.0 ± 0.0
Sham	XI	3.7 ± 0.2	2.0 ± 0.0
Sham	XII	3.8 ± 0.2	2.0 ± 0.0

The results clearly demonstrate that a strong cell-mediated immune response was generated to each of the combinations of purified extracellular proteins. The immunized guinea pigs showed erythema at least twice and usually 3 fold or more that of controls for all combinations. Further, the immunized guinea pigs showed induration at least 3 fold that of controls for all combinations.

Example 18

Immunoprotective Analysis of Combinations III-XII Against Aerosolized *M. tuberculosis*

To demonstrate the prophylactic efficacy of these exemplary combinations of purified extracellular products, guinea pigs immunized with Combinations III through XII were challenged with *M. tuberculosis* three weeks after the last immunization using the protocol of Example 4.

Consistent with earlier results guinea pigs immunized with Combinations III through XII were all protected against death after challenge. At 4 weeks after challenge, 2 of 6 sham-immunized animals (33%) died compared with 0 animals in groups immunized with Combinations IV-XII and 1 of 6 animals (17%) in the group immunized with Combination III. At 10 weeks after challenge, 50% of the sham-immunized animals had died compared with 0 deaths in the animals in groups immunized with Combinations IX and XII (0%), 1 of 6 deaths (17%) in the animals in the groups immunized with Combination III, IV, V, VI, X, and XI, 1 of 5 deaths (20%) in the animals immunized with Combination VIII, and 2 of 6 deaths (33%) in the animals immunized with Combination VII.

Guinea pigs that died before the end of the observation period were autopsied and examined for evidence of gross tuberculosis lesions. Lesions were found in all animals which expired during the study.

Following the conclusion of the mortality study, the surviving animals were sacrificed by hypercarbia and the spleen of each animal was assayed for viable *M. tuberculosis* using the protocol of Example 5. The results are presented in Table R below in terms of mean colony forming units (CFU) along with the log decrease from the sham immunized animals. An asterisk next to the CFU value indicates that spleen counts were zero on one animal in each group. For purposes of calculation, zero counts were treated as 10^3 CFU per spleen or 3 logs.

Table R

Vaccine Group	CFU in Spleen (Mean Log)	Log Decrease from Sham
III	5.99	.5
IV	5.41	1.1
V	6.27	.3
VI	<5.80*	>.7
VII	<5.61*	>.9
VIII	6.47	.1
IX	<5.85*	>.7
X	<5.74*	>.8
XI	5.93	.6
XII	6.03	.5
Sham	6.53	--

Animals immunized with Combinations III, IV, VI, VII, IX, X, XI, and XII had at least 0.5 log fewer colony forming units of *M. tuberculosis* in their spleens on the average than the sham-immunized controls. In particular, combinations IV and VII proved to be especially effective, reducing the average number of colony forming units by roughly a factor of ten. Animals immunized with Combinations V and VIII had 0.3 and 0.1 log fewer colony forming units (CFU), respectively, in their spleens on average, than sham-immunized controls. This dramatic reduction in colony forming units in the animals immunized in accordance with the teachings of the present invention once again illustrates the immunoprotective operability of the present invention.

Example 19

Response of Guinea Pigs Immunized with 3 Different Dosages of Combination XIII to a Challenge with Combination XIII

To further define the operability and scope of the present invention as well as to demonstrate the efficacy of additional combinations of purified extracellular products, guinea pigs were immunized as before using alternative vaccination dosages. Specifically, 50 μg, 100 μg and 200 μg of an alternative combination of 3 majorly abundant extracellular products identified as Combination XIII and comprising the 30 KD, 32(A) KD, and 16 KD proteins. As with the preceding examples, groups of animals were immunized intradermally 4 times, 3 weeks apart with the alternative dosages of Combination XIII in SAF.

A cutaneous hypersensitivity assay was performed to determine if the animals had developed a measurable immune response following vaccination. The animals were shaved over the back and injected intradermally with Combination XIII containing 10.0 μg of each of the purified extracellular products. All injections were performed using a total volume of 0.1 ml. Sham-immunized controls were also skin-tested with the same dosage of Combination XIII. The diameters of erythema and induration at skin- test sites were measured 24 hours after injection.

The results are presented in Table S below in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods

Table S

Vaccine Combination	Vaccine Dose (μg)	Diameter of Skin Reaction (mm) Erythema	Induration
XIII	50	17.8 ± 1.3	13.2 ± 1.0
XIII	100	11.2 ± 0.9	7.3 ± 0.4
XIII	200	10.0 ± 0.7	7.0 ± 0.4
Sham	0	5.7 ± 0.5	0.2 ± 0.2

Once again, these results clearly demonstrate that a strong cell-mediated immune response to Combination XIII was generated in animals immunized with each of the three dosages of Combination XIII. The immunized animals exhibited erythema about two to three times that of controls. Even more strikingly, the immunized animals exhibited induration at least 35 fold that of control animals which exhibited a minimal response in all cases.

Example 20

Immunoprotective Analysis of Combination XIII in Three Different Dosages Against Aerosolized *M. tuberculosis*

To further demonstrate the protective immunity aspects of the vaccines of the present invention at various dosages, the immunized guinea pigs (6 per group) used for the preceding cutaneous hypersensitivity assay were challenged with aerosolized *M. tuberculosis* three weeks after the last immunization. The aerosol challenge was performed using the protocol detailed in Example 4. A control group of 12 sham-immunized animals was challenged simultaneously.

Results of the weekly weight determinations following challenge are graphically represented in Fig. 10 and distinctly show guinea pigs immunized with each of the three dosages of Combination XIII were protected from weight loss. Animals immunized with the higher dosages of Combination XIII (100 and 200 μg) actually showed a net gain in weight and animals immunized with the lower dosage (50 μg) showed a relatively small loss in weight. In contrast, the sham immunized animals lost approximately 22% of their total body weight in the weeks immediately after challenge and averaged a loss of 182 g over the 10 week observation period.

Table U below illustrates the percent weight change for immunized and control animals as determined by taking the mean weight at the end of the challenge, subtracting the mean weight at the start of the challenge and dividing the result by the mean weight at the start of the challenge. Similarly, the percent protection was determined by subtracting the mean percent weight loss of the controls from the mean percent weight gain or loss of the immunized animals.

Table U

Immunogen	Dosage	% Weight Change	% Protection from Weight Loss
Combination XIII	50	-4%	18%
Combination XIII	100	+7%	29%
Combination XIII	200	+5%	27%
Sham	Sham	-22%	-

Table U shows that the sham-immunized animals lost a considerable amount of weight (18% - 29%) over the monitoring period compared with the immunized animals. Fig. 10 provides a more graphic illustration of the net weight loss for each group of immunized animals versus sham-control animals plotted at weekly intervals over the ten week monitoring period. As loss of body mass or "consumption" is one of the classical side effects of tuberculosis, these results indicate that the growth and proliferation of tubercle bacilli in the immunized animals was inhibited by the three different dosages of the exemplary combination vaccine of the present invention.

Example 21

Immunoprotective Analysis of Combinations XIV-XVIII against Challenge with Combinations XIV-XVIII

To further demonstrate the scope of the present invention and the broad range of effective vaccines which may be formulated in accordance with the teachings thereof, five additional combination vaccines, Combinations XIV through XVIII, were tested in guinea pigs. Identified by the apparent molecular weight of the purified extracellular products determined using SDS-PAGE, the composition of each of the combination vaccines is given below.

Combination	Protein Constituents
XIV	30, 32A, 16, 32B, 24, 23, 45
XV	30, 32A, 16, 32B, 24, 23, 45, 23.5, 12
XVI	30, 32A, 16, 32B, 24, 23
XVII	30, 32A, 16, 32B, 24, 71
XVIII	30, 32A, 32B
I	30, 32A, 16, 23, 71

In addition to the new combination vaccines and appropriate controls, Combination I was also used in this series of experiments. Guinea pigs were immunized intradermally with 50 μg of each protein of Combination XIV or XV and with 100 μg of each protein of Combinations I, XVI, XVII, and XVIII all in SAF adjuvant. The animals were immunized a total of four times, with each injection three weeks apart.

A cutaneous hypersensitivity assay was performed to determine if the animals had developed a measurable immune response following vaccination using the previously discussed protocol. Guinea pigs were shaved over the back and injected intradermally with the same combination of purified extracellular proteins to which they were immunized. For each challenge the appropriate combination vaccine containing 10 μg of each protein was injected. All injections were performed using a total volume of 0.1 ml. Sham-immunized controls were also skin-tested with the same dosage of each combination. The diameters of erythema and induration at skin test sites were measured at 24 hours after injection as described in Example 3.

The results of these measurements are presented in Table V below, reported in terms of mean measurement values for the group ± standard error (SE) as determined using traditional methods.

Table V

Vaccine Combination	Skin Test Combination	Diameter of Skin Reaction (mm) Erythema	Induration
XIV	XIV	13.3 ± 0.7	9.1 ± 0.4
XV	XV	10.4 ± 0.4	6.5 ± 0.4
XVI	XVI	8.0 ± 1.8	5.1 ± 1.0
XVII	XVII	9.4 ± 0.9	6.1 ± 1.1
XVIII	XVIII	13.6 ± 1.2	8.7 ± 0.7
I	I	10.0 ± 0.3	6.7 ± 0.2
Sham	XIV	5.5 ± 1.6	0.4 ± 0.2
Sham	XV	6.1 ± 0.5	0.4 ± 0.2
Sham	XVI	4.6 ± 1.4	0.4 ± 0.2
Sham	XVII	5.7 ± 1.2	0.2 ± 0.2
Sham	XVIII	2.1 ± 1.1	0 ± 0
Sham	I	6.0 ± 1.2	0.6 ± 0.2

These results clearly demonstrate that a strong cell-mediated immune response was generated to Combinations XIV through XVIII, and, as before, to Combination I. Immunized animals exhibited erythema about twice that of controls. Even more strikingly, the immunized animals exhibited induration at least 10 fold greater than the sham-immunized controls which exhibited a minimal response in all cases.

Example 22

Immunoprotective Analysis of Combinations XIV-XVIII and Combination I Against Aerosolized *M. tuberculosis*

To confirm the immunoreactivity of the combination vaccines of Example 21 and to demonstrate their applicability to infectious tuberculosis, the immunized guinea pigs used for the preceding cutaneous hypersensitivity assay were challenged with aerosolized *M. tuberculosis* three weeks after the last immunization and monitored using the protocol of Example 4. A control group of 12 sham-immunized animals, the same as used in Example 20, was similarly challenged. The results of these challenge are graphically represented in Fig. 11 and shown in Table W directly below.

Percent weight change was determined by taking the mean weight at the end of the challenge, subtracting the mean weight at the start of the challenge and dividing the result by the mean weight at the start of the challenge. Similarly, the percent protection was determined by subtracting the mean percent weight loss of the controls from the mean percent weight gain or loss of the immunized animals.

Table W

Immunogen	% Weight Change	% Protection from Weight Loss
Combination XIV	3%	25%
Combination XV	- 4%	18%
Combination XVI	-15%	7%
Combination XVII	-11%	11%
Combination XVIII	-12%	10%
Combination I	-11%	11%
Sham	-22%	

As shown in Table W, guinea pigs immunized with each of the combination vaccines were protected from weight loss. Sham-immunized animals lost approximately 22% of their total combined body weight. In contrast the prophylactic effect of the combination vaccines resulted in actual weight gain for one of the test groups and a reduced amount of weight loss in the others. Specifically, animals immunized with Combination XIV evidenced a 3% weight gain while those animals immunized with the other combinations lost only 4% to 15% of their total combined weight.

These results are shown graphically in Fig. 11 which plots weekly weight determinations in terms of net weight gain or loss for each group of animals following aerosolized challenge. This statistically significant difference between the net weight loss for the immunized animals and the sham-immunized controls shown in Fig. 11 provides further evidence for the immunoprophylactic response generated by the combination vaccines of the present invention.

Example 23

Cell-Mediated Immunity in Guinea Pigs Immunized with Three Different Adjuvants

In order to further demonstrate the broad applicability and versatility of the vaccine formulations of the present invention, immunogenic studies were conducted using different adjuvants. Specifically three different immunogens, purified 30 KD protein, Combination I (30, 32A, 16, 23, 71) and Combination XIII (30, 32A, 16) were each formulated using three different adjuvants, Syntex Adjuvant Formulation I (SAF), incomplete Freunds adjuvant (IFA) and Monophosphoryl Lipid A containing adjuvant (MPL). Such adjuvants are generally known to enhance the immune response of an organism when administered with an immunogen.

Guinea pigs were immunized intradermally with 100 μg of each protein comprising Combinations I and XIII and approximately 100 μg of purified 30 KD protein in each of the three different adjuvant formulations. The guinea pigs were immunized with each formulation a total of three times with injections three weeks apart.

Following immunization, a cutaneous hypersensitivity assay was performed to determine if the guinea pigs had developed a measurable immune response. Guinea pigs were shaved over the back and injected intradermally with the same immunogen to which they had been immunized. For the challenge, 10 μg of each protein in Combinations I and XIII or 10 μg of purified 30 KD protein was injected in a total volume of 100 μl. Sham-immunized guinea pigs, vaccinated with one of the three adjuvants, were skin-tested with each of the immunogen formulations containing the same adjuvant. The diameters of erythema and induration at skin test sites were measured 24 hours after challenge as described in Example 3.

The results of these measurements are presented in Table X below. As previously discussed data are reported in terms of mean measurement values for the group ±

standard error as determined using accepted statistical techniques.

Table X

Vaccine	Adjuvant	Skin Test Reagent	Diameter of Skin Reaction (mm)	
			Erythema	Induration
30	SAF	30	10.7 ± 1.6	5.8 ± 1.5
30	IFA	30	8.8 ± 0.7	4.6 ± 0.7
30	MPL	30	10.2 ± 1.7	5.3 ± 1.5
XIII	SAF	XIII	7.3 ± 0.5	4.1 ± 0.5
XIII	IFA	XIII	6.8 ± 0.9	3.5 ± 0.5
XIII	MPL	XIII	6.3 ± 0.4	3.4 ± 0.3
I	SAF	I	6.9 ± 0.6	4.0 ± 0.3
I	IFA	I	6.8 ± 0.2	3.6 ± 0.3
I	MPL	I	7.4 ± 0.4	3.9 ± 0.5
Sham	SAF	30	0.7 ± 0.7	1.0 ± 0
Sham	IFA	30	0 ± 0	0 ± 0
Sham	MPL	30	0 ± 0	0 ± 0
Sham	SAF	XIII	1.0 ± 1.0	1.0 ± 0
Sham	IFA	XIII	0 ± 0	0.3 ± 0.3
Sham	MPL	XIII	0 ± 0	0 ± 0
Sham	SAF	I	4.7 ± 0.3	1.0 ± 0
Sham	IFA	I	2.0 ± 1.0	0.7 ± 0.3
Sham	MPL	I	1.0 ± 1.0	0.7 ± 0.3

As shown in the data presented in Table X, the combination vaccines and purified extracellular products of the present invention provide a strong cell-mediated immunogenic response when formulated with different adjuvants. Moreover, each one of the three adjuvants provided about the same immunogenic response for each respective immunogen. In general, the immunized guinea pigs exhibited erythema diameters approximately seven to ten times that of the sham-immunized guinea pigs while indurations were approximately four to six times greater than measured in the control animals.

The ability of the present invention to provoke a strong immunogenic response in combination with different adjuvants facilitates vaccine optimization. That is, adjuvants used to produce effective vaccine formulations in accordance with the teachings herein may be selected based largely on consideration of secondary criteria such as stability, lack of side effects, cost and ease of storage. These and other criteria, not directly related to the stimulation of an immune response, are particularly important when developing vaccine formulations for widespread use under relatively primitive conditions.

Example 24

Immunoprotective Analysis of Combinations XIX-XXVIII against Challenge with Combinations XIX-XXVIII

The broad scope of the present invention was further demonstrated through the generation of an immune response using ten additional combination vaccines, Combinations XIX through XXVIII. In addition to the new combination vaccines and appropriate controls, Combinations IV and XIII were also used as positive controls to provoke an immune response in guinea pigs. Identified by the apparent molecular weight of the purified extracellular products determined using SDS-PAGE, the composition of each of the combination vaccines is given below.

Combination	Protein Constituents
XIX	30, 32A, 23
XX	30, 32A, 23.5
XXI	30, 32A, 24
XXII	30, 32A, 71
XXIII	30, 32A, 16, 23
XXIV	30, 32A, 16, 23.5
XXV	30, 32A, 16, 24
XXVI	30, 32A, 16, 71
XXVII	30, 32A, 16, 32B
XXVIII	30, 32A, 16, 45
IV	30, 32A
XIII	30, 32A, 16

The guinea pigs were immunized a total of four times, with each injection three weeks apart. Each combination vaccine used to immunize the animals consisted of 100 μg of each protein in SAF adjuvant to provide a total volume of 0.1 ml.

Using the protocol discussed in Example 3, a cutaneous hypersensitive assay was performed to determine if the animals had developed a measurable immune response following vaccination with the selected combination vaccine. The guinea pigs were shaved over the back and injected intradermally with the same combination of purified extracellular proteins with which they were immunized. The protein combinations used to challenge the animals consisted of 10 μg of each protein. Sham immunized controls were also skin-tested with the same dosage of each combination. As in Example 3, the diameters of erythema and induration at the skin test sites were measured at 24 hours after injection.

The results of these measurements are presented in Table Y below, reported in terms of mean measurement values for the group of animals $\pm$ standard error.

Table Y

Vaccine Combination	Skin Test Combination	Diameter of Skin Reaction (mm) Erythema	Induration
XIX	XIX	8.5 ± 0.6	3.9 ± 0.3
XX	XX	8.2 ± 0.3	3.7 ± 0.3
XXI	XXI	11.1 ± 1.1	4.5 ± 0.4
XXII	XXII	9.4 ± 0.8	4.3 ± 0.4
XXIII	XXIII	8.3 ± 1.1	3.0 ± 0.3
XXIV	XXIV	8.5 ± 0.9	3.4 ± 0.5
XXV	XXV	7.9 ± 0.5	3.2 ± 0.4
XXVI	XXVI	8.9 ± 0.7	3.3 ± 0.5
XXVII	XXVII	7.2 ± 1.0	2.8 ± 0.5
XXVIII	XXVIII	8.5 ± 0.5	2.8 ± 0.3
IV	IV	9.0 ± 0.9	4.1 ± 0.3
XIII	XIII	9.4 ± 0.9	4.3 ± 0.3
Sham	XIX	4.0 ± 2.6	1.0 ± 0
Sham	XX	1.3 ± 1.3	1.0 ± 0
Sham	XXI	3.5 ± 1.0	1.3 ± 1.3
Sham	XXII	1.3 ± 1.3	1.0 ± 1.0
Sham	XXIII	0 ± 0	1.0 ± 0
Sham	XXIV	0 ± 0	1.0 ± 0
Sham	XXV	0 ± 0	1.0 ± 0
Sham	XXVI	2.3 ± 2.3	2.0 ± 1.0
Sham	XXVII	0 ± 0	1.0 ± 0
Sham	XXVIII	2.0 ± 1.2	1.0 ± 0
Sham	IV	2.8 ± 1.6	1.0 ± 0
Sham	XIII	1.5 ± 1.5	1.0 ± 0

The results presented in Table Y explicitly show that a strong cell-mediated immune response was generated to Combinations XIX through XXVIII when challenged with the same immunogens. As before, a strong cell-mediated immune response was also provoked by Combinations IV and XIII. The erythema exhibited by the immunized guinea pigs was at least twice, and generally proved to be and more then four fold greater than, the reaction provoked in the corresponding sham immunized control animals. Similarly, the induration exhibited by the immunized animals was at least twice, and generally three to four times greater than, that of the non-immunized controls. The substantially stronger immune response generated among the animals immunized in accordance with the teachings of the present invention once again illustrates the immunoprotective operability of the combination vaccines of the present invention.

Those skilled in the art will also appreciate additional benefits of the vaccines and methods of the present invention. For example, because individual compounds or selected combinations of highly purified molecular species are used for the subject vaccines rather than whole bacteria or components thereof, the vaccines of the present invention are considerably less likely to provoke a toxic response when compared with prior art attenuated or killed bacterial vaccines. Moreover, the molecular vaccines of the present invention are not life threatening to immunocompromised individuals. In fact, the compositions of the present invention may be used therapeutically to stimulate a directed immune response to a pathogenic agent in an infected individual.

Selective use of majorly abundant extracellular products or their immunogenic analogs also prevents the development of an opsonizing humoral response which can increase the pathogenesis of intracellular bacteria. As the protective immunity generated by this invention is directed against unbound proteins, any opsonic response will simply result in the phagocytosis and destruction of the majorly abundant extracellular product rather than the expedited inclusion of the parasitic bacteria. Moreover, the selective use of purified extracellular products reduces the potential for generating a response which precludes the use of widely used screening and control techniques based on host recognition of immunogenic agents. Unlike prior art vaccines, the screening tests could still be performed using an immunoreactive molecule that is expressed by the pathogen but not included in the vaccines made according to the present invention. The use of such an immunogenic determinant would only provoke a response in those individuals which had been exposed to the target pathogen allowing appropriate measures to be taken.

Another advantage of the present invention is that purified extracellular products are easily obtained in large quantities and readily isolated using techniques well known in the art as opposed to the attenuated bacteria and bacterial components of prior art vaccines. Since the immunoreactive products of the present invention are naturally released extracellularly into the surrounding media for most organisms of interest, removal of intracellular contaminants and cellular debris is simplified. Further, as the most prominent or majorly abundant extracellular products or immunogenic analogs thereof are used to stimulate the desired immune response, expression levels and culture concentrations of harvestable product is generally elevated in most production systems. Accordingly, whatever form of production is employed, large scale isolation of the desired products is easily accomplished through routine biochemical procedures such as chromatography or ultrafiltration. These inherent attributes and molecular characteristics of the immunogenic determinants used in the present invention greatly facilitate the production of a consistent, standardized, high quality composition for use on a large scale.

Alternatively, the use of purified molecular compounds based on the immunogenic properties of the most prominent or majorly abundant extracellular products of target pathogens also makes the large scale synthetic generation of the immunoactive vaccine components of the present invention relatively easy. For instance, the extracellular products of interest or their immunogenic analogs may be cloned into a non-pathogenic host bacteria using recombinant DNA technology and harvested in safety. Molecular cloning techniques well known in the art may be used for isolating and expressing DNA corresponding to the extracellular products of interest, their homologs or any segments thereof in selected high expression vectors for insertion in host bacteria such as *Escherichia coli*. Exemplary techniques may be found in II R. Anon, Synthetic

Vaccines 31-77 (1987), Tam et al, *Incorporation of T and B Epitopes of the Circumsporozoite Protein in a Chemically Defined Synthetic Vaccine Against Malaria*, 171 J. Exp. Med. 299-306 (1990), and Stover et al, *Protective Immunity Elicited by Recombinant Bacille Calmette-Guerin (BCG) Expressing Outer Surface Protein A (OspA) Lipoprotein: A Candidate Lyme Disease Vaccine*, 178 J. Exp. Med. 197-209 (1993).

Similarly, the extracellular proteins, their analogs, homologs or immunoreactive protein subunits may be chemically synthesized on a large scale in a relatively pure form using common laboratory techniques and automated sequencer technology. This mode of production is particularly attractive for constructing peptide subunits or lower molecular weight analogs corresponding to antigenic determinants of the extracellular products. Exemplary techniques for the production of smaller protein subunits are well known in the art and may be found in II R. Anon, *Synthetic Vaccines* 15-30 (1987), and in A. Streitwieser, Jr., *Introduction to Organic Chemistry* 953-55 (3rd ed. 1985). Alternative techniques may be found in Gross et al, "Nonenzymatic Cleavage of Peptide Bonds: The Methionine Residues in Bovine Pancreatic Ribonuclease," 237 *The Journal of Biological Chemistry* No. 6 (1962), Mahoney, "High-Yield Cleavage of Tryptophanyl Peptide Bonds by o-Iodosobenzoic Acid," 18 *Biochemistry* No. 17 (1979), and Shoolnik et al, "Gonococcal Pili," 159 *Journal of Experimental Medicine* (1984). Other immunogenic techniques such as anti-idiotyping or directed molecular evolution using peptides, nucleotides or other molecules such as mimetics can also be employed to generate effective, immunoreactive compounds capable of producing the desired prophylactic response. Prior art techniques for the utilization of naked DNA as a vaccine can be found in Robinson, *Protection Against a Lethal Influenza Virus Challenge by Immunization with a Hemagglutinin-Expressing Plasmid DNA*, 11 Vaccine 9 (1993), and in Ulmer et al, *Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein*, 259 Science (1993). Alternatively, techniques for the fusion of a strongly immunogenic protein tail have been disclosed in Tao et al, *Idiotype/Granulocyte-Macrophage Colony-Stimulating Factor Fusion Protein as a Vaccine for B-Ceo Lymphoma*, 362 Nature (1993), and for T-cell epitope mapping in Good et al, *Human T-Cell Recognition of the Circumsporozoite Protein of Plasmodium falciparum: Immunodominant T-Cell Domains Map to the Polymorphic Regions of the Molecule*, 85 Proc. Natl. Acad. Sci. USA (1988), and Gao et al, *Identification and Characterization of T Helper Epitopes in the Nucleoprotein of Influenza A Virus*, 143 The Journal of Immunology No. 9 (1989).

As many bacterial genera exhibit homology, the foregoing examples are provided for the purposes of illustration and are not intended to limit the scope and content of the present invention or to restrict the invention to the genus *Mycobacterium* or to particular species or serogroups therein or to vaccines against tuberculosis alone. It should also be reemphasized that the prevalence of interspecies homology in the DNA and corresponding proteins of microorganisms enables the vaccines of the present invention to induce cross-reactive immunity. Because the immunodominant epitopes of the majorly abundant extracellular products may provide cross-protective immunity against challenge with other serogroups and species of the selected genera, those skilled in the art will appreciate that vaccines directed against one species may be developed using the extracellular products or immunogenic analogs of another species.

For example, *M. bovis* is between 90% and 100% homologous with *M. tuberculosis* and is highly cross-reactive in terms of provoking an immune response. Accordingly, vaccines based on abundant extracellular products of *M. bovis* or other *Mycobacterium* can offer various degrees of protection against infection by *M. tuberculosis* and vice versa. Thus, it is contemplated as being within the scope of the present invention to provide an immunoprophylactic response against several bacterial species of the same genera using an highly homologous immunogenic determinant of an appropriate majorly abundant extracellular product.

It should also be emphasized that the immunogenic determinant selected to practice the present invention may be used in many different forms to elicit an effective immune response. Thus the presentation of one or more immunogenic determinants of selected majorly abundant extracellular products to the host immune system is not critical and may be altered to facilitate production or administration. For example, the vaccines of the present invention may be formulated using whole extracellular products or any immunostimulating fraction thereof including peptides, protein subunits, immunogenic analogs and homologs as noted above. Smaller protein subunits of the majorly abundant extracellular products and molecular analogs thereof are within the scope of the present invention as long as they provoke effective immunoprophylaxis. Moreover, recombinant protein products such as fusion proteins or extracellular products modified through known molecular recombinant techniques are entirely compatible with the teachings of the present invention. In addition, immunogenically generated analogs of the selected immunoactive determinants such as anti-idiotype antibodies, or peptides and nucleotides derived using directed evolution are also within the scope of the invention.

Similarly, the formulation and presentation of the immunogenic agent to the host immune system is not limited to solutions of proteins or their analogs in adjuvant. For example, the immunogenic determinant derived from the appropriate extracellular proteins may be expressed on a different species of bacteria, phage, mycoplasma or virus that is non-pathogenic and modified using recombinant technology. In such cases the whole live organism may be formulated and used to stimulate the desired response.

Conversely, large scale vaccination programs in hostile environments may require very stable formulations without complicating adjuvants or additives. Further, the vaccine formulation could be directed to facilitate the stability or immunoreactivity of the active component when subjected to harsh conditions such as lyophilization or oral administration or encapsulation. Accordingly, the present invention encompasses vastly different formulations of the immunogenic determinants comprising the subject vaccines depending upon the intended use of the product.

Those skilled in the art will appreciate that vaccine dosages should be determined for each pathogen and host utilizing routine experimentation. At present, it is believed that the lowest practical dosage will be on the order of 0.1 μg though dosages of 2.0 μg, 20.0 μg, 100 μg and even 1 mg may be optimum for the appropriate system. The proper dosage can be administered using any conventional immunization technique and sequence known in the art.

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments which have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Horwitz, Marcus A

(ii) TITLE OF INVENTION: Abundant Extracellular Products and Methods for Their Production and Use

(iii) NUMBER OF SEQUENCES: 15

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Ser Lys Val Ser
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Asp Arg Val Ser
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Arg Ala Val Gly
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENTTYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mycobacterium tuberculosis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Glu Lys Thr Pro
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Pro Glu Pro Ala
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Phe Ser Arg Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Ser Arg Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Phe Ser Arg Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Pro Lys Glu Asn
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Pro Lys Thr Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Glu Thr Tyr Leu
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Tyr Pro Ile Thr
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala Asp Pro Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Phe Asp Thr Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Pro Phe Ser Arg Pro Gly Leu Pro Val Glu Tyr Leu Gln Val Pro Ser
1 5 10 15

Asn Ser Met Gly Arg Asp Ile Lys Val Gln Phe Gln Ser Gly Gly Asn
20 25 30

Ser	Pro	Ala	Val	Tyr	Leu	Leu	Asp
		35					40

What is claimed is:

1. A vaccinating agent for use in promoting an effective immune response, in a mammalian host, against an infectious pathogen from the genus *Mycobacterium*, said vaccinating agent comprising:

at least one majorly abundant extracellular product selected from the group consisting of *M. tuberculosis* 110 KD protein, 80 KD protein, 71 KD protein, 58 KD protein, 45 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23.5 KD protein, 23 KD protein, 16 KD protein, 14 KD protein and 12 KD protein.

2. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 110 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5    10    15    20
NSKSV NSFGA HDTLK V-ERK RQ
```

written left to right in the direction of the amino terminus to the carboxy terminus.

3. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 80 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5
TDRVS VGN
```

written left to right in the direction of the amino terminus to the carboxy terminus.

4. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 71 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5
ARAVG I
```

written left to right in the direction of the amino terminus to the carboxy terminus.

5. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 58 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5     10     15     20
TEKTP  DDVFK  LAKDE  KVLYL
```

written left to right in the direction of the amino terminus to the carboxy terminus.

6. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 45 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5     10     15     20     25     30
DPEPA  PPVPD  DAASP  PDDAA  APPAP  ADPP-
```

written left to right in the direction of the amino terminus to the carboxy terminus.

7. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 32B KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5     10     15     20
FSRPG  LPVEY  LQVPS  A-MGR  DI
```

written left to right in the direction of the amino terminus to the carboxy terminus.

8. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 32A KD protein or an immunoreactive homolog

or fragment thereof having an N-terminal amino acid sequence of

```
    5    10    15    20    25    30    35
FSRPG LPVEY LQVPS PSMGR DIKVQ FQSGG ANSP-

   40
LYLLD
```

written left to right in the direction of the amino terminus to the carboxy terminus.

9. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 30 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5    10    15    20    25    30    35
FSRPG LPVEY LQVPS PSMGR DIKVQ FQSGG NNSPA

   40
VYLLD
```

written left to right in the direction of the amino terminus to the carboxy terminus.

10. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 24 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5    10    15    20    25    30    35
APYEN LMVPS PSMGR DIPVA FLAGG PHAVY LLDAF

   40    45    50    55     60
NAGPD VSNWV TAGNA MMTLA -KGIC/S
```

written left to right in the direction of the amino terminus to the carboxy terminus.

11. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 23.5 KD protein or an immunoreactive homolog

or fragment thereof having an N-terminal amino acid sequence of

```
    5      10
APKTY  -EELK  GTD
```

written left to right in the direction of the amino terminus to the carboxy terminus.

12. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 23 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5     10     15     20
AETYL  PDLDW  DYGAL  EPHIS  GQ
```

written left to right in the direction of the amino terminus to the carboxy terminus.

13. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 16 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

```
    5     10     15     20     25     30
AYPIT  GKLGS  ELTMT  DTVGQ  VVLGW  KVSDL

     35     40     45
F/YKSTA  VIPGY  TV-EQ  QI
```

written left to right in the direction of the amino terminus to the carboxy terminus.

14. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 14 KD protein or an immunoreactive homolog or

fragment thereof having an N-terminal amino acid sequence of

5	10	15	20	25	30
ADPRL	QFTAT	TLSGA	PFDGA	S/NLQ<u>G</u>K	PAVL<u>W</u>

written left to right in the direction of the amino terminus to the carboxy terminus.

15. The vaccinating agent of claim 1 wherein said at least one majorly abundant extracellular product is *M. tuberculosis* 12 KD protein or an immunoreactive homolog or fragment thereof having an N-terminal amino acid sequence of

5	10	15	20	25	30	35
FDTRL	MRLED	EMKEG	RYEVR	AELPG	VDPDK	DVDIM
40	45					
VRDGQ	LTIKA	ERT				

written left to right in the direction of the amino terminus to the carboxy terminus.

16. The vaccinating agent of claim 1 wherein said at least one compound sufficiently analogous to at said least one majorly abundant extracellular product of said pathogen is selected from the group of analogs consisting of peptides, homologs, fusion proteins, glycosylates and immunologically acceptable salts thereof.

17. The vaccinating agent of claim 1 further comprising an adjuvant composition.

18. A substantially pure, majorly abundant extracellular protein product of *M. tuberculosis*, or immunoreactive analog or homolog thereof, capable of promoting an immune response in a mammalian host.

19. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the

protein product has an apparent molecular weight of approximately 12 KD as determined by SDS-PAGE and has an N-terminal amino acid sequence of

5 10 15 20 25 30 35
FDTRL MRLED EMKEG RYEVR AELPG VDPDK DVDIM

40 45
VRDGQ LTIKA ERT

written left to right in the direction of the amino terminus to the carboxy terminus.

20. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of approximately 14 KD as determined by SDS-PAGE and has an N-terminal amino acid sequence of

5 10 15 20 25 30
ADPRL QFTAT TLSGA PFDGA S/NLQGK PAVLW

written left to right in the direction of the amino terminus to the carboxy terminus.

21. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of approximately 16 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

5 10 15 20 25 30
AYPIT GKLGS ELTMT DTVGQ VVLGW KVSDL

35 40 45
F/YKSTA VIPGY TV-EQ QI

written left to right in the direction of the amino terminus to the carboxy terminus.

22. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the

protein product has an apparent molecular weight of approximately 24 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

5	10	15	20	25	30	35
APYEN	LMVPS	PSMGR	DIPVA	FLAGG	PHAVY	LLDAF
40	45	50	55	60		
NAGPD	VSNWV	TAGNA	MMTLA	-KGIC/S		

written left to right in the direction of the amino terminus to the carboxy terminus.

23. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of approximately 45 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

5	10	15	20	25	30
DPEPA	PPVPD	DAASP	PDDAA	APPAP	ADPP-

written left to right in the direction of the amino terminus to the carboxy terminus.

24. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of approximately 58 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

5	10	15	20
TEKTP	DDVFK	LAKDE	KVLYL

written left to right in the direction of the amino terminus to the carboxy terminus.

25. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of

approximately 80 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

```
    5
TDRVS  VGN
```

written left to right in the direction of the amino terminus to the carboxy terminus.

26. The substantially pure, majorly abundant extracellular *M. tuberculosis* protein product of claim 18, or immunoreactive analog or homolog thereof, wherein the protein product has an apparent molecular weight of approximately 110 KD as determined by SDS-PAGE and an N-terminal amino acid sequence of

```
    5      10     15     20
NSKSV  NSFGA  HDTLK  V-ERK  RQ
```

written left to right in the direction of the amino terminus to the carboxy terminus.

27. A combination vaccine for use in promoting an effective immune response in a mammalian host against an infectious pathogen of the genus *Mycobacterium*, said combination vaccine comprising:

a plurality of majorly abundant extracellular products selected from the group consisting of *M. tuberculosis* 110 KD protein, 80 KD protein, 71 KD protein, 58 KD protein, 45 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23.5 KD protein, 23 KD protein, 16 KD protein, 14 KD protein and 12 KD protein.

28. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein, 24 KD protein, 23 KD protein and 16 KD protein.

29. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 32B KD protein, 30 KD protein, 23 KD protein and 16 KD protein.

30. The combination vaccine of claim 20 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein and 30 KD protein.

31. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32B KD protein and 30 KD protein.

32. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 30 KD protein and 16 KD protein.

33. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 30 KD protein and 23 KD protein.

34. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 71 KD protein and 30 KD protein.

35. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 30 KD protein and 23.5 KD protein.

36. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 30 KD protein and 12 KD protein.

37. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 30 KD protein and 24 KD protein.

38. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 58 KD protein and 30 KD protein.

39. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 45 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23 KD protein and 16 KD protein.

40. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 45 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23.5 KD protein, 23 KD protein, 16 KD protein, and 12 KD protein.

41. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23 KD protein and 16 KD protein.

42. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 71 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein and 16 KD protein.

43. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 32B KD protein and 30 KD protein.

44. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of M. tuberculosis 32A KD protein, 30 KD protein and 16 KD protein.

45. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein and 23 KD protein.

46. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein and 23.5 KD protein.

47. The combination vaccine of claim 20 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein and 24 KD protein.

48. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein and 71 KD protein.

49. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein, 23 KD and 16 KD protein.

50. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein, 23.5 KD protein and 16 KD protein.

51. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 30 KD protein, 24 KD protein and 16 KD protein.

52. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 71 KD protein, 32A KD protein, 30 KD protein and 16 KD protein.

53. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 32A KD protein, 32B KD protein, 30 KD protein and 16 KD protein.

54. The combination vaccine of claim 27 wherein said combination vaccine includes a mixture of *M. tuberculosis* 45 KD protein, 32A KD protein, 30 KD protein and 16 KD protein.

55. A method for immunizing a mammalian host against an infectious pathogen of the genus *Mycobacterium*, said method comprising the steps of:

providing a combination vaccine of a mixture of compounds sufficiently analogous to a plurality of majorly abundant extracellular products, said majorly abundant extracellular products selected from the group consisting of *M. tuberculosis* 110 KD protein, 80 KD protein, 71 KD protein, 58 KD protein, 45 KD protein, 32A KD protein, 32B KD protein, 30 KD protein, 24 KD protein, 23.5 KD protein, 23 KD protein, 16 KD protein, 14 KD protein and 12 KD protein to have the capacity to stimulate an effective immune response to subsequent infection by said pathogen; and

administering a prophylactically effective amount of said combination vaccine to said mammalian host.

56. The method of claim 55 wherein said infectious pathogen is selected from the group consisting of *M. tuberculosis, M. bovis, M. marinum, M.kansasii, M. avium-intracellulare, M. fortuitum, M. chelonei, M. scrofulaceum, M. leprae, M. africanum, M. ulcerans* and *M. microti*.

57. The method of claim 55 wherein said plurality of compounds sufficiently analogous to said plurality of majorly abundant extracellular products of said pathogen are synthetically produced.

58. The method of claim 55 further comprising the step of formulating said combination vaccine with an adjuvant composition prior to said administrating step.

59. The method of claim 55 wherein said mammalian host is a human.

60. The method of claim 55 wherein said mammalian host is a domesticated animal selected from the group consisting of dogs, cats, cattle, sheep, horses and pigs.

FIG. 1d.

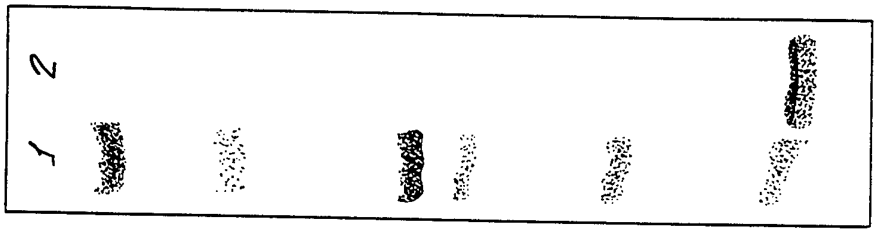


FIG. 1c.

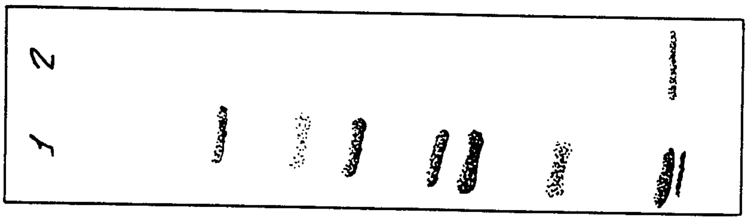


FIG. 1b.

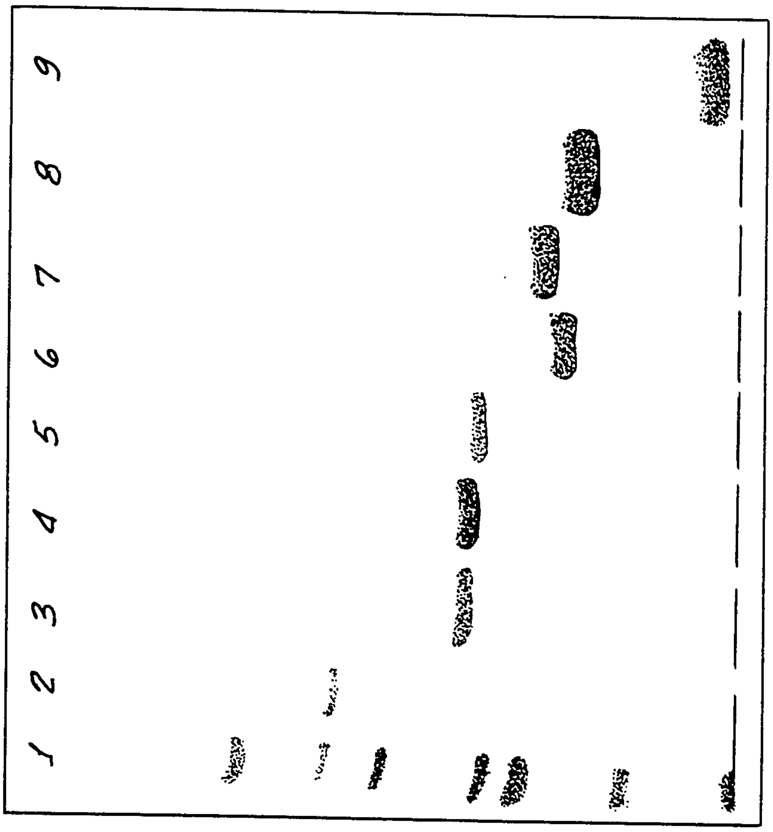


FIG. 1a.

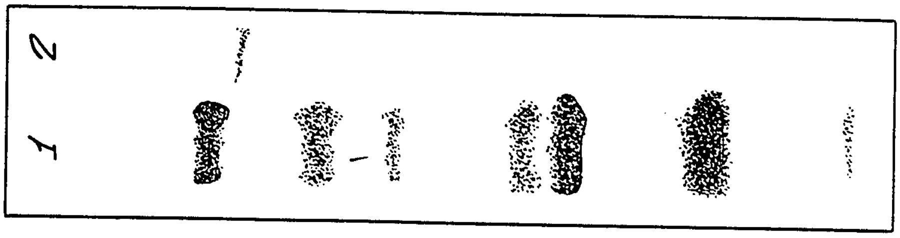


FIG. 2.

PURIFIED EXTRACELLULAR PROTEINS STUDIED	
APPARENT MW BY SDS-PAGE (KD)	N TERMINAL 5 AMINO ACIDS
110	NSKSV
80	TDRVS
*71	ARAVG
58	TEKTP
45	DPEPA
*32A	FSRPG
32B	FSRPG
*30	FSRPG
24	APYEN
23.5	APKTY
*23	AETYL
*16	AYPIT
14	ADPRL
12	FDTRL

FIG. 3.

EXTENDED N-TERMINAL SEQUENCE OF 30/32 KD COMPLEX OF M. TUBERCULOSIS EXTRACELLULAR PROTEINS

	1									10										20
30	F	S	R	P	G	L	P	V	E	Y	L	Q	V	P	S	P	S	M	G	R
32A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-

	21									30										40
30	D	I	K	V	Q	F	Q	S	G	G	N	N	S	P	A	V	Y	L	L	D
32A	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	L	-	-	-	-
32B	-	-																		

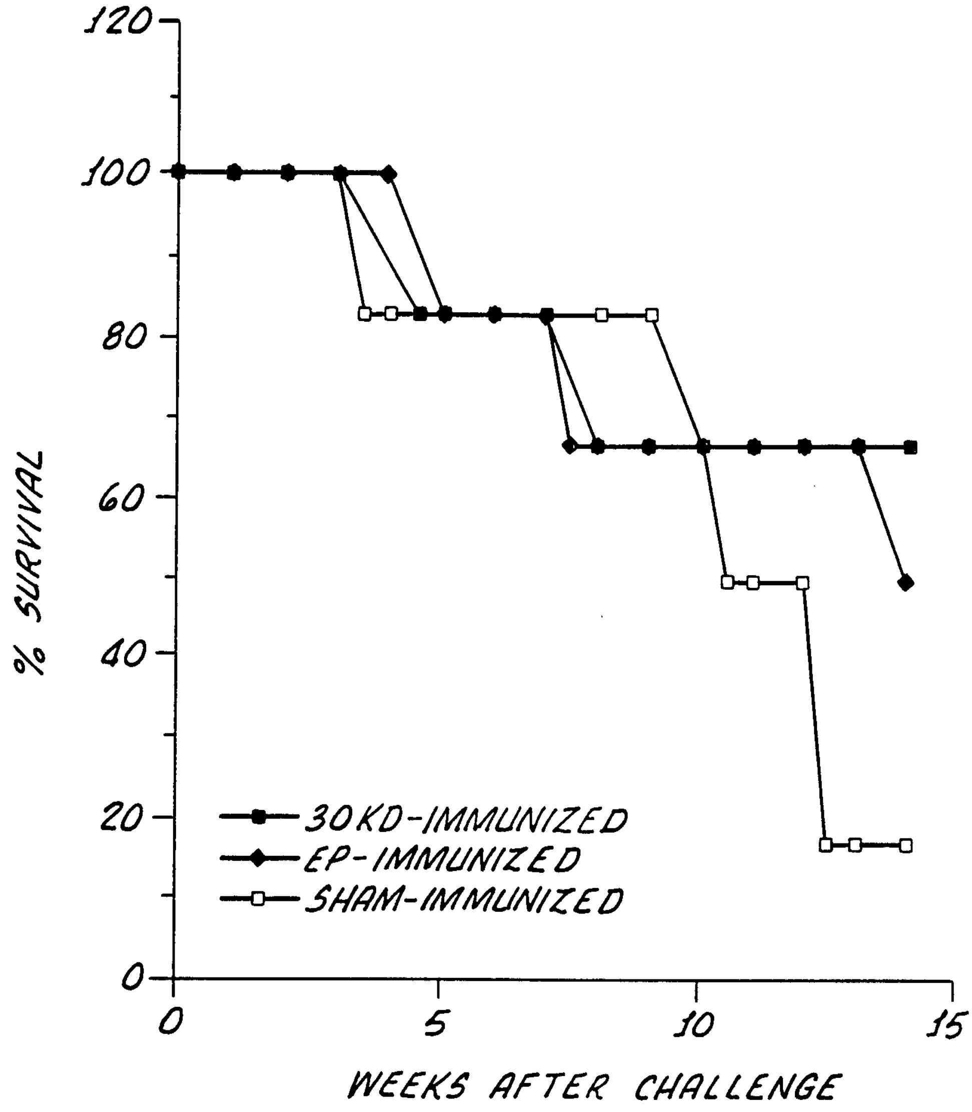
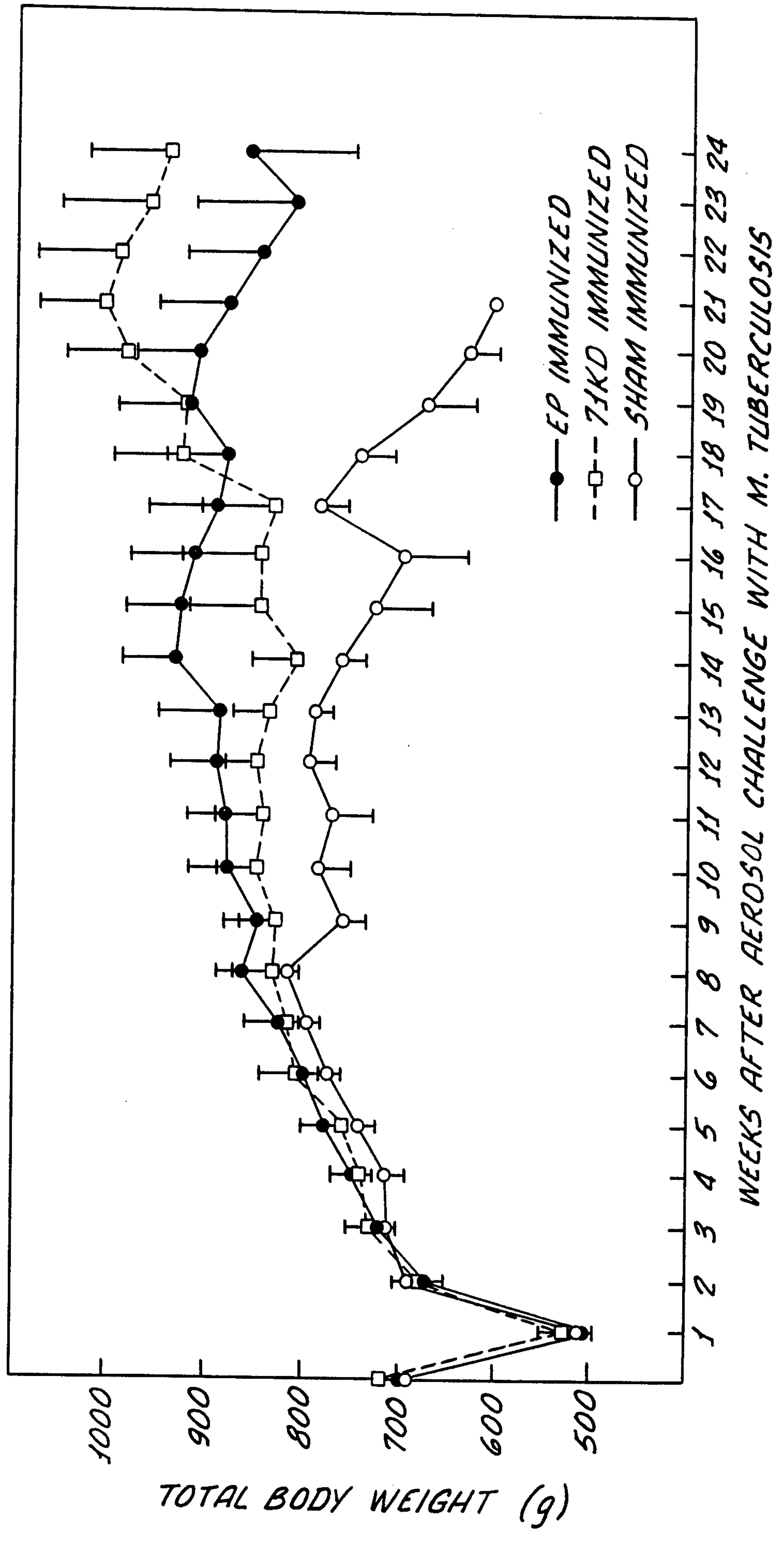

120
100
80
60
40
20
0
% SURVIVAL
0
5
10
15
WEEKS AFTER CHALLENGE
30KD-IMMUNIZED
EP-IMMUNIZED
SHAM-IMMUNIZED

FIG. 4.

FIG. 5.


TOTAL BODY WEIGHT (g)
1000
900
800
700
600
500
WEEKS AFTER AEROSOL CHALLENGE WITH M. TUBERCULOSIS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
EP IMMUNIZED
71KD IMMUNIZED
SHAM IMMUNIZED

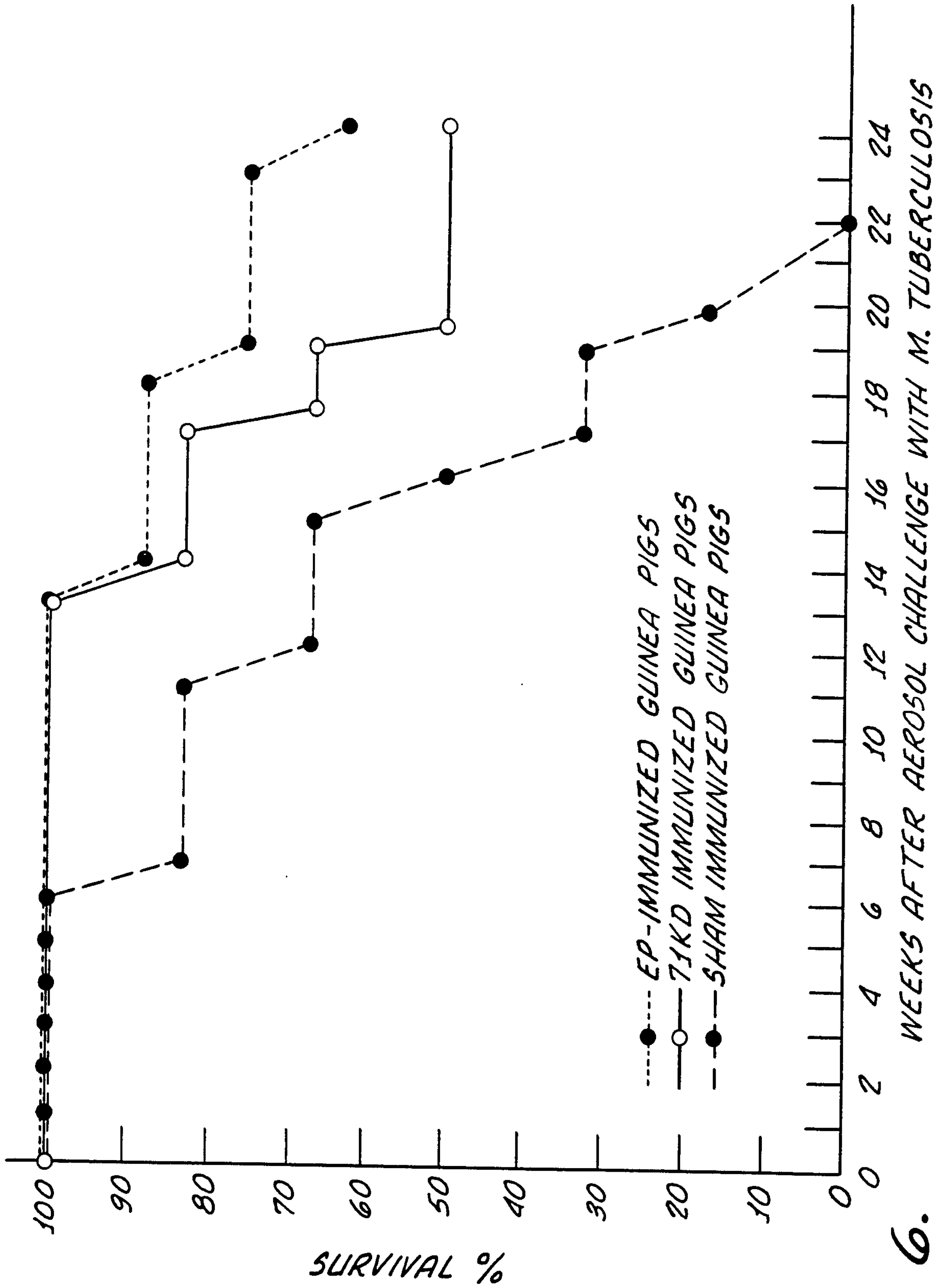

EP-IMMUNIZED GUINEA PIGS
71KD IMMUNIZED GUINEA PIGS
SHAM IMMUNIZED GUINEA PIGS
SURVIVAL %
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
WEEKS AFTER AEROSOL CHALLENGE WITH M. TUBERCULOSIS

FIG. 6.

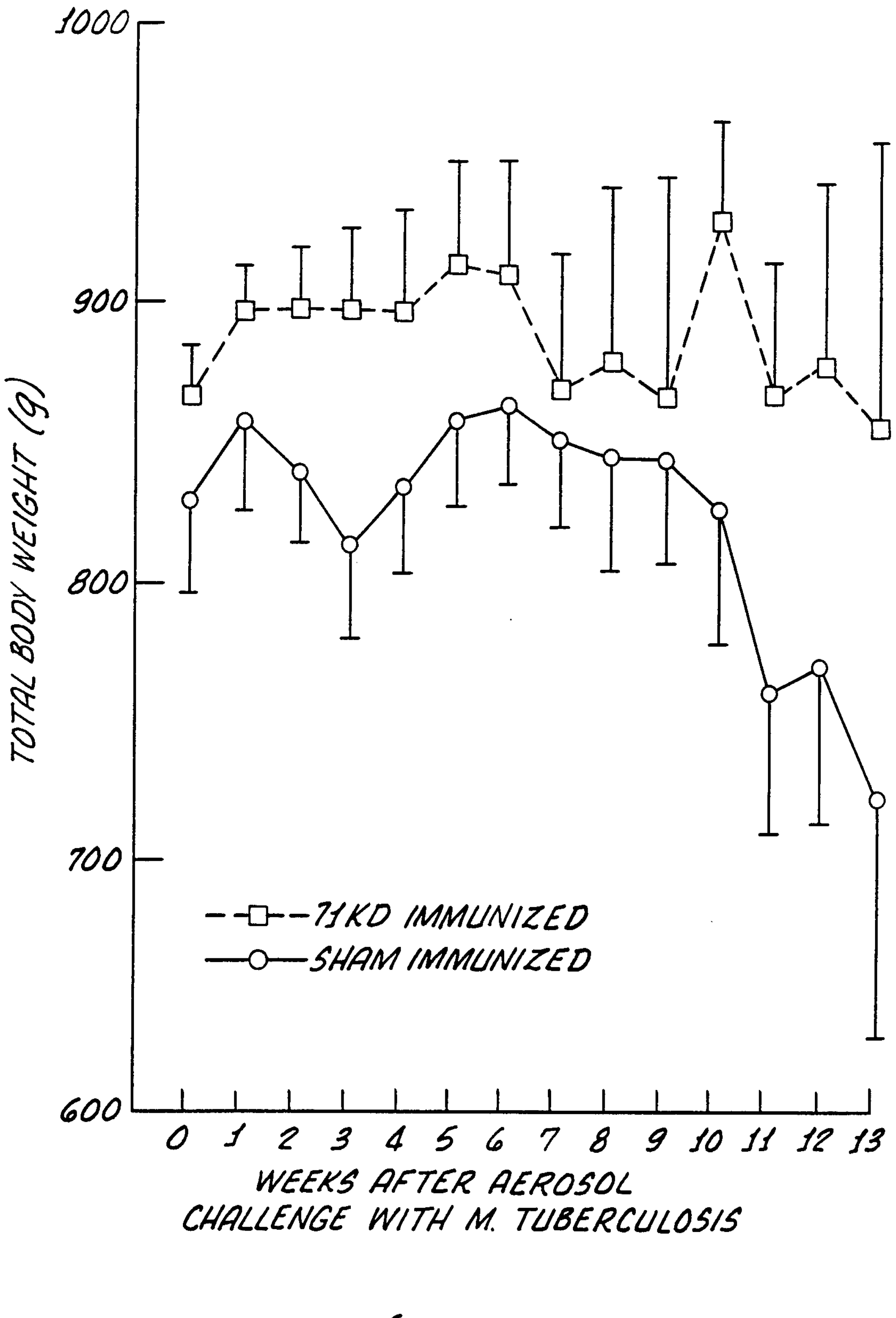

1000
900
800
700
600
TOTAL BODY WEIGHT (g)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
WEEKS AFTER AEROSOL
CHALLENGE WITH M. TUBERCULOSIS
71KD IMMUNIZED
SHAM IMMUNIZED

FIG. 7.

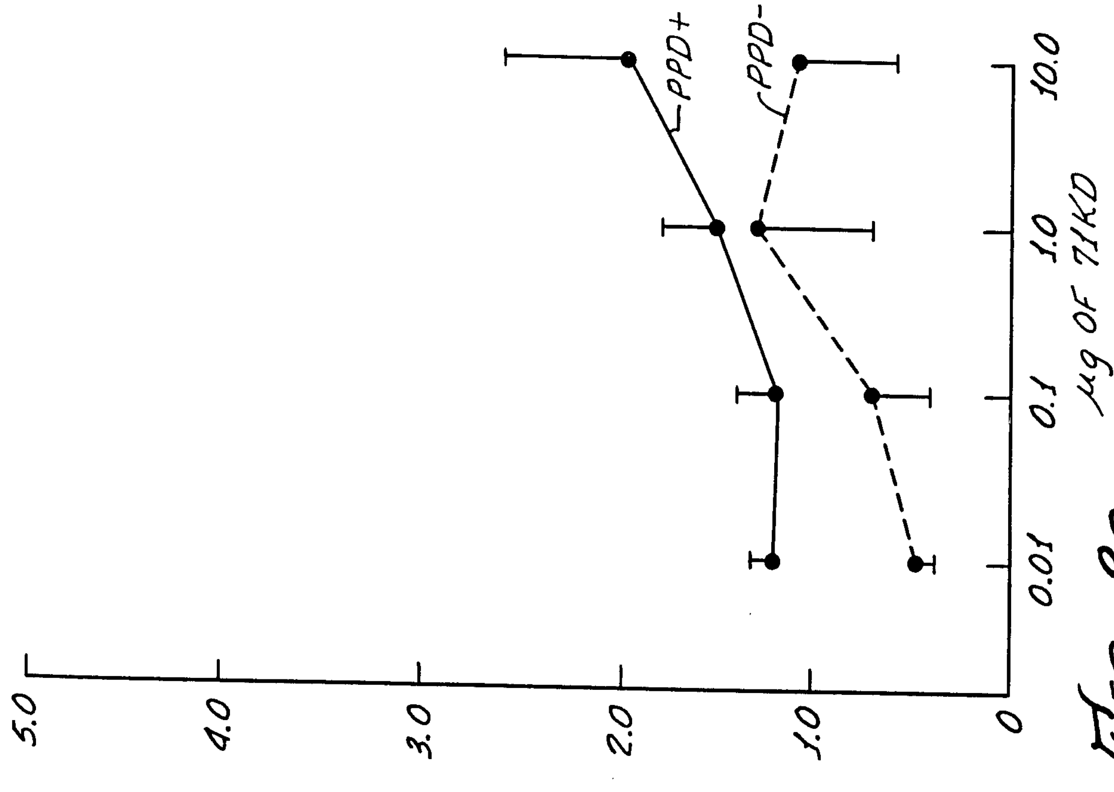

5.0
4.0
3.0
2.0
1.0
0
0.01
0.1
1.0
10.0
μg OF 71KD
PPD+
PPD-

FIG. 8a.

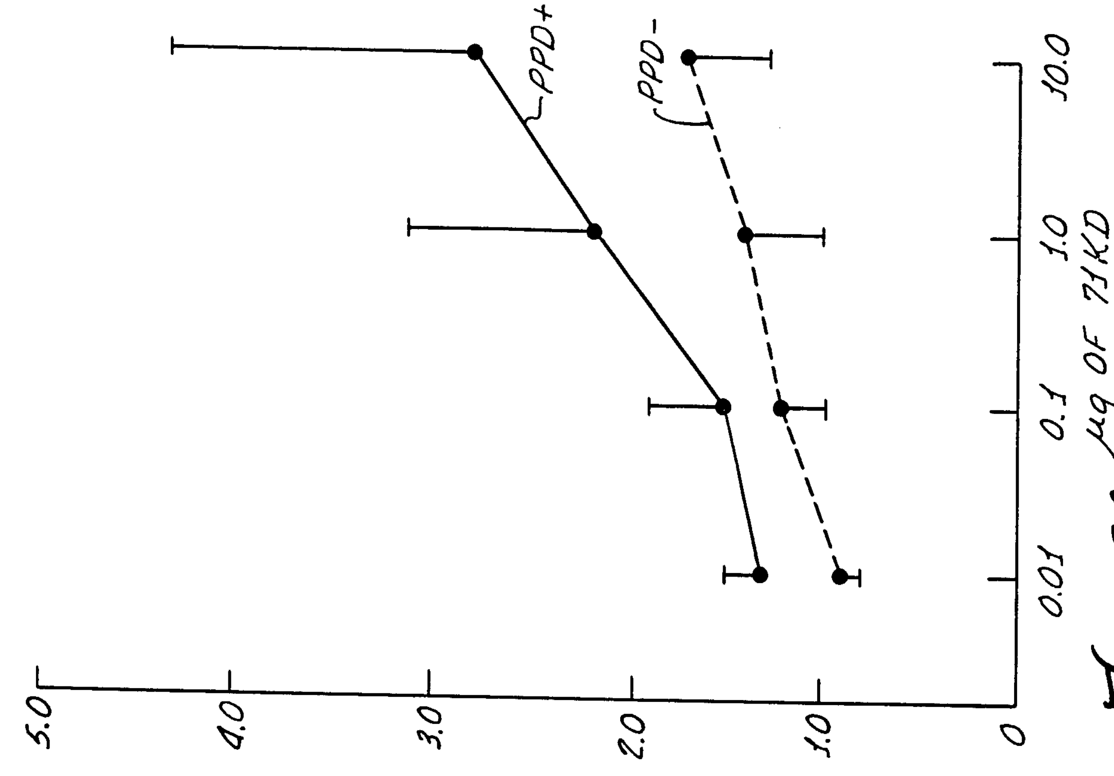

5.0
4.0
3.0
2.0
1.0
0
0.01
0.1
1.0
10.0
μg OF 71KD
PPD+
PPD-

FIG. 8b.

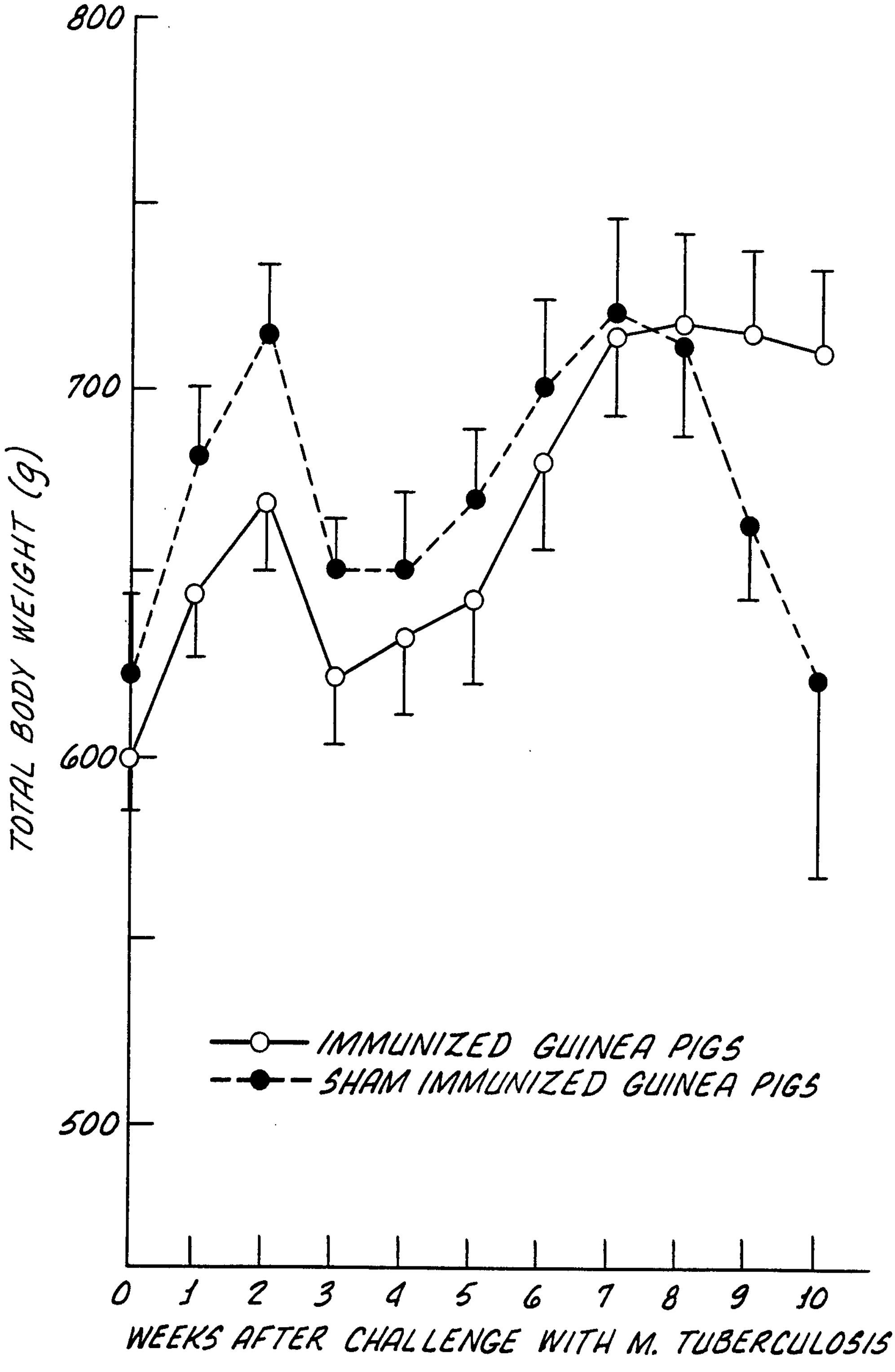

800
700
600
500
TOTAL BODY WEIGHT (g)
0 1 2 3 4 5 6 7 8 9 10
WEEKS AFTER CHALLENGE WITH M. TUBERCULOSIS
IMMUNIZED GUINEA PIGS
SHAM IMMUNIZED GUINEA PIGS

FIG. 9.

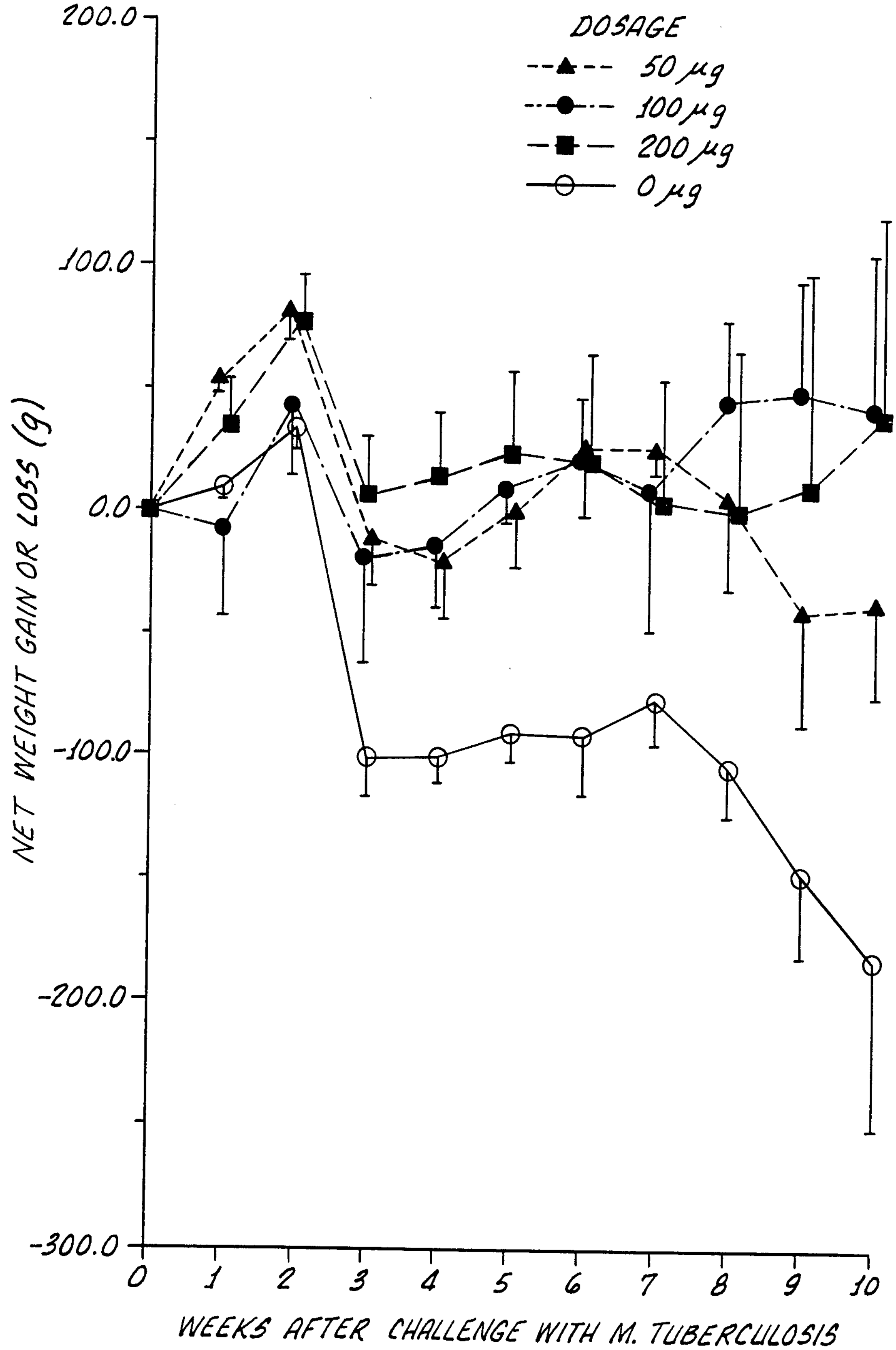

DOSAGE
50 μg
100 μg
200 μg
0 μg
200.0
100.0
0.0
-100.0
-200.0
-300.0
NET WEIGHT GAIN OR LOSS (g)
0
1
2
3
4
5
6
7
8
9
10
WEEKS AFTER CHALLENGE WITH M. TUBERCULOSIS

FIG. 10.

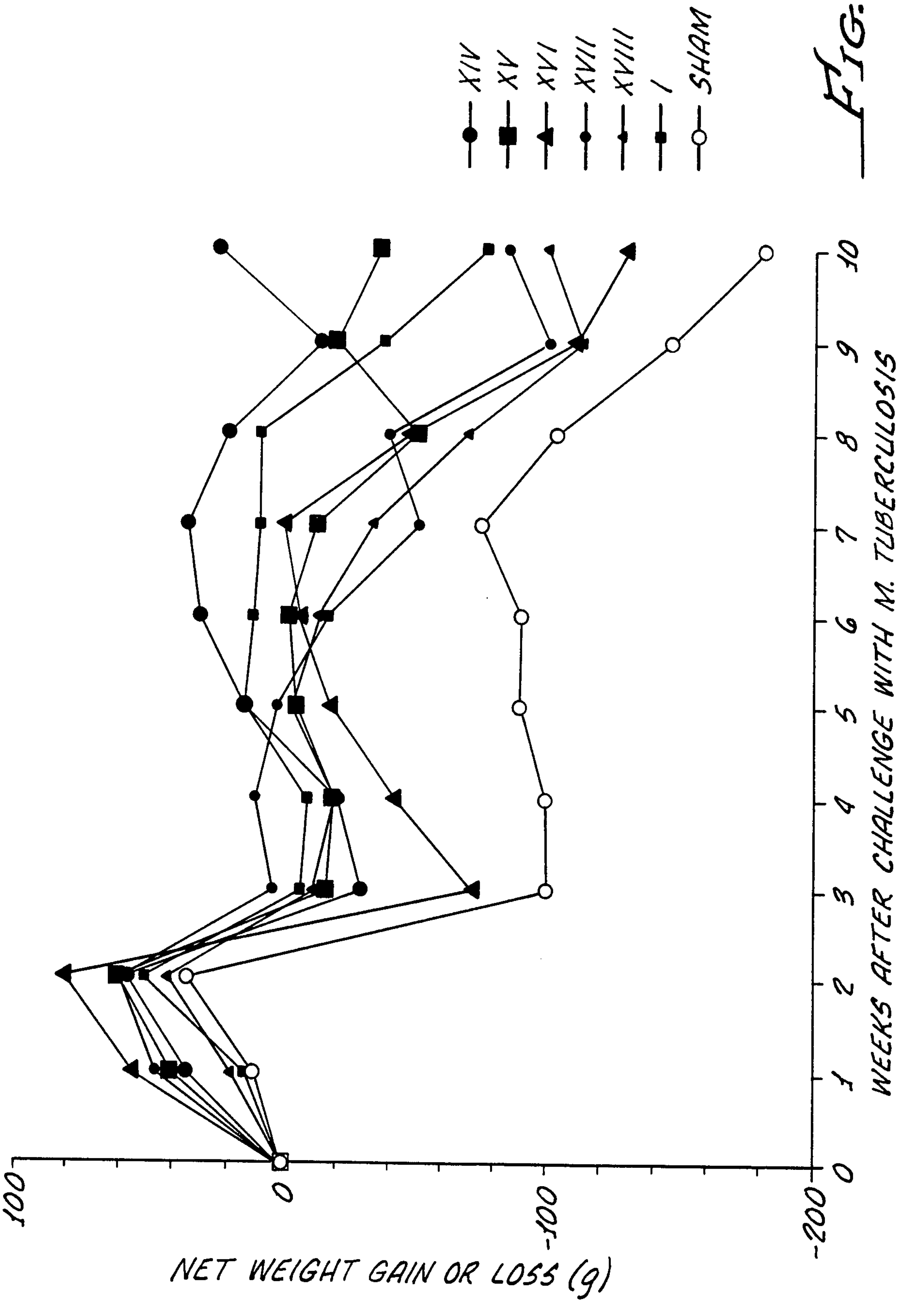

XIV
XV
XVI
XVII
XVIII
I
SHAM
NET WEIGHT GAIN OR LOSS (g)
100
0
-100
-200
WEEKS AFTER CHALLENGE WITH M. TUBERCULOSIS
0
1
2
3
4
5
6
7
8
9
10

FIG. 11.