COMPOSITIONS DERIVED FROM MYCOBACTERIUM VACCAE AND METHODS FOR THEIR USE

Technical Field

The present invention relates generally to compositions which are present in or may be derived from *Mycobacterium vaccae* and their use in the treatment, prevention and detection of disorders including infectious diseases, immune disorders and cancer. In particular, the invention is related to compounds and methods for the treatment of diseases of the respiratory system, such as mycobacterial infections, asthma, sarcoidosis and lung cancers, and disorders of the skin, such as psoriasis, atopic dermatis, allergic contact dermatitis, alopecia areata, and the skin cancers basal cell carcinoma, squamous cell carcinoma and melanoma. The invention is further related to compounds that function as non-specific immune response amplifiers, and the use of such non-specific immune response amplifiers as adjuvants in vaccination or immunotherapy against infectious disease, and in certain treatments for immune disorders and cancer.

Background of the Invention

Tuberculosis is a chronic, infectious disease, that is caused by infection with *Mycobacterium tuberculosis (M. tuberculosis)*. It is a major disease in developing countries, as well as an increasing problem in developed areas of the world, with about 8 million new cases and 3 million deaths each year. Although the infection may be asymptomatic for a considerable period of time, the disease is most commonly manifested as a chronic inflammation of the lungs, resulting in fever and respiratory symptoms. If left untreated, significant morbidity and death may result.

Although tuberculosis can generally be controlled using extended antibiotic therapy, such treatment is not sufficient to prevent the spread of the disease. Infected individuals may be asymptomatic, but contagious, for some time. In addition, although compliance with the

treatment regimen is critical, patient behaviour is difficult to monitor. Some patients do not complete the course of treatment, which can lead to ineffective treatment and the development of drug resistant mycobacteria.

Inhibiting the spread of tuberculosis requires effective vaccination and accurate, early diagnosis of the disease. Currently, vaccination by subcutaneous or intradermal injection with live bacteria is the most efficient method for inducing protective immunity. The most common mycobacterium employed for this purpose is Bacillus Calmette-Guerin (BCG), an avirulent strain of *Mycobacterium bovis* (*M. bovis*). However, the safety and efficacy of BCG is a source of controversy and some countries, such as the United States, do not vaccinate the general public. Diagnosis of *M. tuberculosis* infection is commonly achieved using a skin test, which involves intradermal exposure to tuberculin PPD (protein-purified derivative). Antigen-specific T cell responses result in measurable induration at the injection site by 48-72 hours after injection, thereby indicating exposure to mycobacterial antigens. Sensitivity and specificity have, however, been a problem with this test, and individuals vaccinated with BCG cannot be distinguished from infected individuals.

A less well-known mycobacterium that has been used for immunotherapy for tuberculosis and also leprosy, by subcutaneous or intradermal injection, is *Mycobacterium vaccae* (*M. vaccae*), which is non-pathogenic in humans. However, there is less information on the efficacy of *M. vaccae* compared with BCG, and it has not been used widely to vaccinate the general public. *M. bovis* BCG and *M. vaccae* are believed to contain antigenic compounds that are recognised by the immune system of individuals exposed to infection with *M. tuberculosis*.

Several patents and other publications disclose treatment of various conditions by administering mycobacteria, including *M. vaccae*, or certain mycobacterial fractions. U.S. Patent 4,716,038 discloses diagnosis of, vaccination against and treatment of autoimmune diseases of various types, including arthritic diseases, by administering mycobacteria, including *M. vaccae*. U.S. Patent 4,724,144 discloses an immunotherapeutic agent comprising antigenic material derived from *M. vaccae* for treatment of mycobacterial diseases, especially tuberculosis and leprosy, and as an adjuvant to chemotherapy.

International Patent Publication WO 91/01751 discloses the use of antigenic and/or immunoregulatory material from *M. vaccae* as an immunoprophylactic to delay and/or prevent the onset of AIDS. International Patent Publication WO 94/06466 discloses the use of antigenic and/or immunoregulatory material derived from *M. vaccae* for therapy of HIV infection, with or without AIDS and with or without associated tuberculosis.

U.S. Patent 5,599,545 discloses the use of mycobacteria, especially whole, inactivated *M. vaccae*, as an adjuvant for administration with antigens which are not endogenous to *M. vaccae*. This publication theorises that the beneficial effect as an adjuvant may be due to heat shock protein 65 (hsp 65). International Patent Publication WO 92/08484 discloses the use of antigenic and/or immunoregulatory material derived from *M. vaccae* for the treatment of uveitis. International Patent Publication WO 93/16727 discloses the use of antigenic and/or immunoregulatory material derived from *M. vaccae* for the treatment of mental diseases associated with an autoimmune reaction initiated by an infection. International Patent Publication WO 95/26742 discloses the use of antigenic and/or immunoregulatory material derived from *M. vaccae* for delaying or preventing the growth or spread of tumors. International Patent Publication WO 91/02542 discloses the use of autoclaved *M. vaccae* in the treatment of chronic inflammatory disorders in which a patient demonstrates an abnormally high release of IL-6 and/or TNF or in which the patient's IgG shows an abnormally high proportion of agalactosyl IgG. Among the disorders mentioned in this publication are psoriasis, rheumatoid arthritis, mycobacterial disease, Crohn's disease, primary biliary cirrhosis, sarcoidosis, ulcerative colitis, systemic lupus erythematosus, multiple sclerosis, Guillain-Barre syndrome, primary diabetes mellitus, and some aspects of graft rejection.

M. vaccae is apparently unique among known mycobacterial species in that heat-killed preparations retain vaccine and immunotherapeutic properties. For example, *M. tuberculosis* BCG vaccines, used for vaccination against tuberculosis, employ live strains. Heat-killed *M. bovis* BCG and *M. tuberculosis* have no protective properties when employed in vaccines. A number of compounds have been isolated from a range of mycobacterial

species which have adjuvant properties. The effect of such adjuvants is essentially to stimulate a particular immune response mechanism against an antigen from another species.

There are two general classes of compounds which have been isolated from mycobacterial species that exhibit adjuvant properties. The first are water soluble wax D fractions (R.G. White, I. Bernstock, R.G.S. Johns and E. Lederer, Immunology, 1:54, 1958; US Patent 4,036,953). The second are muramyl dipeptide-based substances (N-acetyl glucosamine and N-glycolymuramic acid in approximately equimolar amounts) as described in U.S. Patents 3,956,481 and 4,036,953. These compounds differ from the delipidated and deglycolipidated *M. vaccae* (DD-*M. vaccae*) of the present invention in the following aspects of their composition:

1. They are water-soluble agents, whereas DD-*M. vaccae* is insoluble in aqueous solutions.
2. They consist of a range of small oligomers of the mycobacterial cell wall unit, either extracted from bacteria by various solvents, or digested from the cell wall by an enzyme. In contrast, DD-*M. vaccae* contains highly polymerised cell wall.
3. All protein has been removed from their preparations by digestion with proteolytic enzymes. The only constituents of their preparations are the components of the cell wall peptidoglycan structure, namely alanine, glutamic acid, diaminopimelic acid, N-acetyl glucosamine, and N-glycolylmuramic acid. In contrast, DD-*M. vaccae* contains 50% w/w protein, comprising a number of distinct protein species.

The delivery of vaccines by nasal aerosols to reach lung tissue, or by oral delivery to the gastrointestinal tract has been generally limited to attenuated strains of virus. For example, vaccination against poliovirus has employed oral delivery of attenuated strains of this virus since the development of the Sabin vaccine. Aviron Incorporated and the National Institute of Allergy and Infectious Diseases in the United States have recently reported the

successful use of an influenza vaccine administered in a nasal spray. In this case, a live attenuated influenza strain provided 93% protection against influenza in young children. Vaccines consisting of killed viruses or bacteria, or of recombinant proteins have not been delivered by nasal aerosol or oral delivery. There are several reasons for this. There are few reports of successful immunisation resulting in T cell immunity or antibody synthesis employing these agents administered nasally. Further, oral delivery of proteins and killed organisms often results in the development of tolerance, which is exactly the reverse outcome sought in successful immunisation.

Sarcoidosis is a disease of unknown cause characterised by granulomatous inflammation affecting many organs of the body and especially the lungs, lymph nodes and liver. Sarcoid granulomata are composed of mononuclear phagocytes, with epithelioid and giant cells in their centre, and T lymphocytes. CD4 T lymphocytes are closely associated with the epithelioid cells while both CD4 and CD8 T lymphocytes accumulate at the periphery. The characteristic immunological abnormalities in sarcoidosis include peripheral blood and bronchoalveolar lavage hyper-globulinaemia and depression of 'delayed type' hypersensitivity reactions in the skin to tuberculin and other similar antigens, such as *Candida* and mumps. Peripheral blood lymphocyte numbers are reduced and CD4: CD8 ratios in peripheral blood are depressed to approximately 1-1.5:1. These are not manifestations of a generalised immune defect, but rather the consequence of heightened immunological activity which is 'compartmentalised' to sites of disease activity. In patients with pulmonary sarcoidosis, the total number of cells recovered by bronchoalveolar lavage is increased five- to ten-fold and the proportion of lymphocytes increased from the normal of less than 10-14% to between 15% and 50%. More than 90% of the lymphocytes recovered are T lymphocytes and the CD4:CD8 ratio has been reported to be increased from the value of 1.8:1 in normal controls to 10.5:1. The T lymphocytes are predominantly of the Th1 class, producing IFN-γ and IL-2 cytokines, rather than of the Th2 class. Following treatment, the increase in Th1 lymphocytes in sarcoid lungs is corrected.

Sarcoidosis involves the lungs in nearly all cases. Even when lesions are predominantly seen in other organs, subclinical lung involvement is usually present. While

some cases of sarcoidosis resolve spontaneously, approximately 50% of patients have at least a mild degree of permanent organ dysfunction. In severe cases, lung fibrosis develops and progresses to pulmonary failure requiring lung transplantation. The mainstay of treatment for sarcoidosis is corticosteroids. Patients initially responding to corticosteroids often relapse and require treatment with other immunosuppressive drugs such as methotrexate or cyclosporine.

Asthma is a common disease, with a high prevalence in the developed world. Asthma is characterised by increased responsiveness of the tracheobronchial tree to a variety of stimuli, the primary physiological disturbance being reversible airflow limitation, which may be spontaneous or drug-related, and the pathological hallmark being inflammation of the airways. Clinically, asthma can be subdivided into extrinsic and intrinsic variants.

Extrinsic asthma has an identifiable precipitant, and can be thought of as being atopic, occupational and drug-induced. Atopic asthma is associated with the enhancement of a Th2-type of immune response with the production of specific immunoglobulin E (IgE), positive skin tests to common aeroallergens and/or atopic symptoms. It can be divided further into seasonal and perennial forms according to the seasonal timing of symptoms. The airflow obstruction in extrinsic asthma is due to nonspecific bronchial hyperesponsiveness caused by inflammation of the airways. This inflammation is mediated by chemicals released by a variety of inflammatory cells including mast cells, eosinophils and lymphocytes. The actions of these mediators result in vascular permeability, mucus secretion and bronchial smooth muscle constriction. In atopic asthma, the immune response producing airway inflammation is brought about by the Th2 class of T cells which secrete IL-4, IL-5 and IL-10. It has been shown that lymphocytes from the lungs of atopic asthmatics produce IL-4 and IL-5 when activated. Both IL-4 and IL-5 are cytokines of the Th2 class and are required for the production of IgE and involvement of eosinophils in asthma. Occupational asthma may be related to the development of IgE to a protein hapten, such as acid anhydrides in plastic workers and plicatic acid in some western red cedar-induced asthma, or to non-IgE related mechanisms, such as that seen in toluene diisocyanate-induced asthma. Drug-induced asthma can be seen after the administration of aspirin or other non-steroidal anti-inflammatory drugs, most often in a certain subset of patients who may display other features such as nasal

polyposis and sinusitis. Intrinsic or cryptogenic asthma is reported to develop after upper respiratory tract infections, but can arise *de novo* in middle-aged or older people, in whom it is more difficult to treat than extrinsic asthma.

Asthma is ideally prevented by the avoidance of triggering allergens but this is not always possible nor are triggering allergens always easily identified. The medical therapy of asthma is based on the use of corticosteroids and bronchodilator drugs to reduce inflammation and reverse airway obstruction. In chronic asthma, treatment with corticosteroids leads to unacceptable adverse side effects.

Another disorder with a similar immune abnormality to asthma is allergic rhinitis. Allergic rhinitis is a common disorder and is estimated to affect at least 10% of the population. Allergic rhinitis may be seasonal (hay fever) caused by allergy to pollen. Non-seasonal or perennial rhinitis is caused by allergy to antigens such as those from house dust mite or animal dander.

The abnormal immune response in allergic rhinitis is characterised by the excess production of IgE antibodies specific against the allergen. The inflammatory response occurs in the nasal mucosa rather than further down the airways as in asthma. Like asthma, local eosinophilia in the affected tissues is a major feature of allergic rhinitis. As a result of this inflammation, patients develop sneezing, nasal discharge and congestion. In more severe cases, the inflammation extends to the eyes (conjunctivitis), palate and the external ear. While it is not life threatening, allergic rhinitis may be very disabling, prevent normal activities, and interfere with a person's ability to work. Current treatment involves the use of antihistamines, nasal decongestants and, as for asthma, sodium cromoglycate and corticosteroids.

Lung cancer is the leading cause of death from cancer. The incidence of lung cancer continues to rise and the World Health Organisation estimates that by 2000AD there will be 2 million new cases annually. Lung cancers may be broadly classified into two categories: small cell lung cancer (SCLC) which represents 20-25% of all lung cancers, and non-small cell lung cancer (NSCLC) which accounts for the remaining 75%. The majority of SCLC is caused by tobacco smoke. SCLC tends to spread early and 90% of patients present at diagnosis with involvement of the mediastinal lymph nodes in the chest. SCLC is treated by

chemotherapy, or a combination of chemotherapy and radiotherapy. Complete response rates vary from 10% to 50%. For the rare patient without lymph node involvement, surgery followed by chemotherapy may result in cure rates exceeding 60%. The prognosis for NSCLC is more dismal, as most patients have advanced disease by the time of diagnosis. Surgical removal of the tumor is possible in a very small number of patients and the five year survival rate for NSCLC is only 5-10%.

The factors leading to the development of lung cancer are complex and multiple. Environmental and genetic factors interact and cause sequential and incremental abnormalities which lead to uncontrolled cell proliferation, invasion of adjacent tissues and spread to distant sites.

Both cell-mediated and humoral immunity have been shown to be impaired in patients with lung cancer. Radiotherapy and chemotherapy further impair the immune function of patients. Attempts have been made to immunise patients with inactivated tumour cells or tumour antigens to enhance host anti-tumor response. Bacillus Calmette-Guerin (BCG) has been administered into the chest cavity following lung cancer surgery to augment non-specific immunity. Attempts have been made to enhance anti-tumor immunity by giving patients lymphocytes treated *ex vivo* with interleukin-2. These lymphokine-activated lymphocytes acquire the ability to kill tumor cells. The current immunotherapies for lung cancer are still at a developmental stage and their efficacies yet to be established for the standard management of lung cancer.

In one aspect, this invention deals with treatment of disorders of skin which appear to be associated with factors that influence the balance of thymus-derived (T) immune cells known as Th1 and Th2. These T cells are identified by their cytokine secretion phenotype. A common feature of treatment is the use of compounds prepared from *M. vaccae* which have immunomodulating properties that alter the balance of activities of these T cells as well as other immune cells.

Psoriasis is a common, chronic inflammatory skin disease which can be associated with various forms of arthritis in a minority of patients. The defect in psoriasis appears to be overly rapid growth of keratinocytes and shedding of scales from the skin surface. Drug

therapy is directed at slowing down this process. The disease may become manifest at any age. Spontaneous remission is relatively rare, and life-long treatment is usually necessary. Psoriasis produces chronic, scaling red patches on the skin surface. Psoriasis is a very visible disease, it frequently affects the face, scalp, trunk and limbs. The disease is emotionally and physically debilitating for the patient, detracting significantly from the quality of life. Between one and three million individuals in the United States have psoriasis with nearly a quarter million new cases occurring each year. Conservative estimates place the costs of psoriasis care in the United States currently at $248 million a year.

There are two major hypotheses concerning the pathogenesis of psoriasis. The first is that genetic factors determine abnormal proliferation of epidermal keratinocytes. The cells no longer respond normally to external stimuli such as those involved in maintaining epidermal homeostasis. Abnormal expression of cell membrane cytokine receptors or abnormal transmembrane signal transduction might underlie cell hyperproliferation. Inflammation associated with psoriasis is secondary to the release of pro-inflammatory molecules from hyperproliferative keratinocytes.

A second hypothesis is that T cells interacting with antigen-presenting cells in skin release pro-inflammatory and keratinocyte-stimulating cytokines (Hancock, G.E. et al., *J. Exp. Med. 168*:1395-1402, 1988). Only T cells of genetically predetermined individuals possess the capacity to be activated under such circumstances. The keratinocytes themselves may be the antigen-presenting cell. The cellular infiltrate in psoriatic lesions show an influx of CD4+ T cells and, more prominently, CD8+ T cells (Bos, J.D. et al., *Arch. Dermatol. Res. 281*:23-3, 1989; Baker, B.S., *Br. J. Dermatol. 110*:555-564, 1984).

As the majority (90%) of psoriasis patients have limited forms of the disease, topical treatments which include dithranol, tar preparations, corticosteroids and the recently introduced vitamin D3 analogues (calcipotriol, calcitriol) can be used. A minority (10%) of psoriasis patients have a more serious condition, for which a number of systemic therapeutic modalities are available. Specific systemic therapies include UVB, PUVA, methotrexate, vitamin A derivatives (acitretin) and immuno-suppressants such as Cyclosporin A. The effectiveness of Cyclosporin and FK-506 for treating psoriasis provides support for the T cell

hypothesis as the prime cause of the disease (Bos, J.D. et al., *Lancet II*: 1500-1502, 1989; Ackerman, C. et al., *J. Invest. Dermatol. 96*:536 [abstract], 1991).

Atopic dermatitis is a chronic pruritic inflammatory skin disease which usually occurs in families with an hereditary predisposition for various allergic disorders such as allergic rhinitis and asthma. Atopic dermatitis occurs in approximately 10% of the general population. The main symptoms are dry skin, dermatitis (eczema) localised mainly in the face, neck and on the flexor sides and folds of the extremities accompanied by severe itching. It typically starts within the first two years of life. In about 90% of the patients this skin disease disappears during childhood but the symptoms can continue into adult life. It is one of the commonest forms of dermatitis world-wide. It is generally accepted that in atopy and in atopic dermatitis, a T cell abnormality is primary and that the dysfunction of T cells which normally regulate the production of IgE is responsible for the excessive production of this immunoglobulin.

Allergic contact dermatitis is a common non-infectious inflammatory disorder of the skin. In contact dermatitis, immunological reactions cannot develop until the body has become sensitised to a particular antigen. Subsequent exposure of the skin to the antigen and the recognition of these antigens by T cells result in the release of various cytokines, proliferation and recruitment of T cells, and finally in dermatitis (eczema).

Only a small proportion of the T cells in a lesion of allergic contact dermatitis are specific for the relevant antigen. Activated T cells probably migrate to the sites of inflammation regardless of antigen-specificity. Delayed-type hypersensitivity can only be transferred by T cells ($CD4^+$ cells) sharing the MHC class II antigens. The 'response' to contact allergens can be transferred by T cells sharing either MHC class I ($CD8^+$ cells) or class II ($CD4^+$ cells) molecules (Sunday, M.E. et al., *J. Immunol. 125*:1601-1605, 1980). Keratinocytes can produce interleukin-1 which can facilitate the antigen presentation to T cells. The expression of the surface antigen intercellular adhesion molecule-1 (ICAM-1) is induced both on keratinocytes and endothelium by the cytokines tumor necrosis factor (TNF) and interferon-gamma (IFN-γ).

If the causes can be identified, removal alone will cure allergic contact dermatitis. During active inflammation, topical corticosteroids are useful. An inhibitory effect of cyclosporin has been observed in delayed-type hypersensitivity on the pro-inflammatory function(s) of primed T cells *in vitro* (Shidani, B. et al., *Eur. J. Immunol. 14*:314-318, 1984). The inhibitory effect of cyclosporin on the early phase of T cell activation in mice has also been reported (Milon, G. et al., *Ann. Immunol. (Inst. Pasteur) 135d*: 237-245, 1984).

Alopecia areata is a common hair disease, which accounts for about 2% of the consultations at dermatological outpatient clinics in the United States. The hallmark of this disease is the formation of well-circumscribed round or oval patches of non-scarring alopecia which may be located in any hairy area of the body. The disease may develop at any age. The onset is usually sudden and the clinical course is varied.

At present, it is not possible to attribute all or indeed any case of alopecia areata to a single cause (Rook, A. and Dawber, R, Diseases of the Hair and Scalp; Blackwell Scientific Publications 1982: 272-30). There are many factors that appear to be involved. These include genetic factors, atopy, association with disorders of supposed autoimmune etiology, Down's syndrome and emotional stress. The prevalence of atopy in patients with alopecia areata is increased. There is evidence that alopecia areata is an autoimmune disease. This evidence is based on consistent histopathological findings of a lymphocytic T cell infiltrate in and around the hair follicles with increased numbers of Langerhans cells, the observation that alopecia areata will respond to treatment with immunomodulating agents, and that there is a statistically significant association between alopecia areata and a wide variety of autoimmune diseases (Mitchell, A.J. et al., *J. Am. Acad. Dermatol. 11*:763-775, 1984).

Immunophenotyping studies on scalp biopsy specimens shows expression of HLA-DR on epithelial cells in the presumptive cortex and hair follicles of active lesions of alopecia areata, as well as a T cell infiltration with a high proportion of helper/inducer T cells in and around the hair follicles, increased numbers of Langerhans cells and the expression of ICAM-1 (Messenger, A.G. et al., *J. Invest. Dermatol. 85*:569-576, 1985; Gupta, A.K. et al., *J. Am. Acad. Dermatol. 22*:242-250, 1990).

The large variety of therapeutic modalities in alopecia areata can be divided into four categories: (i) non-specific topical irritants; (ii) 'immune modulators' such as systemic corticosteroids and PUVA; (iii) 'immune enhancers' such as contact dermatitis inducers, cyclosporin and inosiplex; and (iv) drugs of unknown action such as minoxidil (Dawber, R.P.R. et al., Textbook of Dermatology, Blackwell Scientific Publications, 5th Ed, 1982:2533-2638). Non-specific topical irritants such as dithranol may work through as yet unidentified mechanisms rather than local irritation in eliciting regrowth of hair. Topical corticosteroids may be effective but prolonged therapy is often necessary. Intralesional steroids have proved to be more effective but their use is limited to circumscribed patches of less active disease or to maintain regrowth of the eyebrows in alopecia totalis. Photochemotherapy has proved to be effective, possibly by changing functional subpopulations of T cells. Topical immunotherapy by means of induction and maintenance of allergic contact dermatitis on the scalp may result in hair regrowth in as many as 70% of the patients with alopecia areata. Diphencyprone is a potent sensitiser free from mutagenic activity. Oral cyclosporin can be effective in the short term (Gupta, A.K. et al., *J. Am. Acad. Dermatol. 22*:242-250, 1990). Inosiplex, an immunostimulant, has been used with apparent effectiveness in an open trial. Topical 5% minoxidil solution has been reported to be able to induce some hair growth in patients with alopecia areata. The mechanism of action is unclear.

Carcinomas of the skin are a major public health problem because of their frequency and the disability and disfigurement that they cause. Carcinoma of the skin is principally seen in individuals in their prime of life, especially in fair skinned individuals exposed to large amounts of sunlight. The annual cost of treatment and time loss from work exceeds $250 million dollars a year in the United States alone. The three major types - basal cell cancer, squamous cell cancer, and melanoma - are clearly related to sunlight exposure.

Basal cell carcinomas are epithelial tumours of the skin. They appear predominantly on exposed areas of the skin. In a recent Australian study, the incidence of basal cell carcinomas was 652 new cases per year per 100,000 of the population. This compares with 160 cases of squamous cell carcinoma or 19 of malignant melanoma (Giles, G. et al., *Br. Med. J. 296*:13-17, 1988). Basal cell carcinomas are the most common of all cancers.

Lesions are usually surgically excised. Alternate treatments include retinoids, 5-fluorouracil, cryotherapy and radiotherapy. Alpha or gamma interferon have also been shown to be effective in the treatment of basal cell carcinomas, providing a valuable alternative to patients unsuitable for surgery or seeking to avoid surgical scars (Cornell et al., *J. Am. Acad. Dermatol. 23*:694-700, 1990; Edwards, L. et al., *J. Am. Acad. Dermatol. 22*:496-500, 1990).

Squamous cell carcinoma (SCC) is the second most common cutaneous malignancy, and its frequency is increasing. There are an increasing number of advanced and metastatic cases related to a number of underlying factors. Currently, metastatic SCC contributes to over 2000 deaths per year in the United States; the 5 year survival rate is 35%, with 90% of the metastases occurring by 3 years. Metastasis almost always occurs at the first lymphatic drainage station. The need for medical therapy for advanced cases is clear. A successful medical therapy for primary SCC of the skin would obviate the need for surgical excision with its potential for scarring and other side effects. This development may be especially desirable for facial lesions.

Because of their antiproliferative and immunomodulating effects *in vitro*, interferons (IFNs) have also been used in the treatment of melanoma (Kirkwood, J.M. et al., *J. Invest. Dermatol. 95*:180S-4S, 1990). Response rates achieved with systemic IFN-α, in either high or low dose, in metastatic melanoma were in the range 5-30%. Recently, encouraging results (30% response) were obtained with a combination of IFN-α and DTIC. Preliminary observations indicate a beneficial effect of IFN-α in an adjuvant setting in patients with high risk melanoma. Despite the low efficacy of IFN monotherapy in metastatic disease, several randomised prospective studies are now being performed with IFNs as an adjuvant or in combination with chemotherapy (McLeod, G.R. et al., *J. Invest. Dermatol. 95*:185S-7S, 1990; Ho, V.C. et al., *J. Invest. Dermatol.* 22:159-76, 1990).

Of all the available therapies for treating cutaneous viral lesions, only interferon possesses a specific antiviral mode of action, by reproducing the body's immune response to infection. Interferon treatment cannot eradicate the viruses however, although it may help with some manifestations of the infection. Interferon treatment is also associated with systemic adverse effects, requires multiple injections into each single wart and has a

significant economic cost (Kraus, S.J. et al., *Review of Infectious Diseases* 2(6):S620-S632, 1990; Frazer, I.H., *Current Opinion in Immunology* 8(4):484-491, 1996).

Summary of the Invention

Briefly stated, the present invention provides compositions present in or derived from *M. vaccae* and methods for their use in the prevention, treatment and diagnosis of diseases, including mycobacterial infection, immune disorders of the respiratory system, and skin disorders. The inventive methods comprise administering a composition having antigenic and/or adjuvant properties. Diseases of the respiratory system which may be treated using the inventive compositions include mycobacterial infections (such as infection with *M. tuberculosis* and/or *M. avium*), asthma, sarcoidosis and lung cancers. Disorders of the skin which may be treated using the inventive compositions include psoriasis, atopic dermatis, allergic contact dermatitis, alopecia areata, and the skin cancers basal cell carcinoma, squamous cell carcinoma and melanoma. Adjuvants for use in vaccines or immunotherapy of infectious diseases and cancers are also provided.

In a first aspect, isolated polypeptides derived from *Mycobacterium vaccae* are provided comprising an immunogenic portion of an antigen, or a variant of such an antigen. In specific embodiments, the antigen includes an amino acid sequence selected from the group consisting of: (a) sequences recited in SEQ ID NO: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207; (b) sequences having at least about 50% identical residues to a sequence recited in SEQ ID NO: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207; (c) sequences having at least about 75% identical residues to a sequence recited in SEQ ID NO: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207; and (d) sequences having at least about 95% identical residues to a sequence recited in SEQ ID NO: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196,

197, 199, 201, 203, 205 and 207, measured using alignments produced by the computer algorithm BLASTP, as described below.

DNA sequences encoding the inventive polypeptides, expression vectors comprising these DNA sequences, and host cells transformed or transfected with such expression vectors are also provided. In another aspect, the present invention provides fusion proteins comprising at least one polypeptide of the present invention.

Within other aspects, the present invention provides pharmaceutical compositions that comprise at least one of the inventive polypeptides, or a DNA molecule encoding such a polypeptide, and a physiologically acceptable carrier. The invention also provides vaccines comprising at least one of the above polypeptides, or at least one DNA sequence encoding such polypeptides, and a non-specific immune response amplifier. In certain embodiments, the non-specific immune response enhancer is selected from the group consisting of: delipidated and deglycolipidated *M. vaccae* cells; inactivated *M. vaccae* cells; delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids; delipidated and deglycolipidated M.vaccae cells depleted of mycolic acids and arabinogalactan; and *M. vaccae* culture filtrate.

In yet another aspect, methods are provided for enhancing an immune response in a patient, comprising administering to a patient an effective amount of one or more of the above pharmaceutical compositions and/or vaccines. In one embodiment, the immune response is a Th1 response. In further aspects of this invention, methods are provided for the treatment of a disorder in a patient, comprising administering to the patient a pharmaceutical composition or vaccine of the present invention. In certain embodiments, the disorder is selected from the group consisting of immune disorders, infectious diseases, skin diseases and diseases of the respiratory system. Examples of such diseases include mycobacterial infections, asthma and psoriasis.

In other aspects, the invention provides methods for the treatment of immune disorders, infectious diseases, skin diseases or diseases of the respiratory system, comprising administering a composition comprising inactivated *M. v*accae cells, delipidated and deglycolipidated *M. vaccae* cells or *M. vaccae* culture filtrate.

Methods for enhancing an immune response to an antigen are also provided. In one embodiment, such methods comprising administering a polypeptide that comprises an immunogenic portion of a *M. vaccae* antigen which includes a sequence of SEQ ID NO: 89 or 201, or a variant thereof. In a further embodiment, such methods comprise administering a composition comprising a component selected from the group consisting of: delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids, and delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids and arabinogalactan.

In further aspects of this invention, methods and diagnostic kits are provided for detecting mycobacterial infection in a patient. In a first embodiment, the method comprises contacting dermal cells of a patient with one or more of the above polypeptides and detecting an immune response on the patient's skin. In a second embodiment, the method comprises contacting a biological sample with at least one of the above polypeptides; and detecting in the sample the presence of antibodies that bind to the polypeptide or polypeptides, thereby detecting *M. tuberculosis* infection in the biological sample. Suitable biological samples include whole blood, sputum, serum, plasma, saliva, cerebrospinal fluid and urine.

Diagnostic kits comprising one or more of the above polypeptides in combination with an apparatus sufficient to contact the polypeptide with the dermal cells of a patient are provided. The present invention also provides diagnostic kits comprising one or more of the inventive polypeptides in combination with a detection reagent.

In yet another aspect, the present invention provides antibodies, both polyclonal and monoclonal, that bind to the polypeptides described above, as well as methods for their use in the detection of *mycobacterial* infection.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

Brief Description of the Drawings

Figs. 1A and 1B illustrate the protective effects of immunizing mice with autoclaved *M. vaccae* or unfractionated *M. vaccae* culture filtrates, respectively, prior to infection with live *M. tuberculosis* H37Rv.

Figs. 2A and B show the percentage of eosinophils in mice immunized intranasally with either 10 or 1000 μg of heat-killed *M. vaccae* or 200-100 μg of DD-*M. vaccae*, respectively, 4 weeks prior to challenge with ovalbumin, as compared to control mice. Figs. 2C and D show the percentage of eosinophils in mice immunized intranasally with either 100 μg of heat-killed *M. vaccae* or 200 μg of DD-*M. vaccae*, respectively, as late as one week prior to challenge with ovalbumin. Fig. 2E shows the percentage of eosinophils in mice immunized either intranasally (i.n.) or subcutaneously (s.c.) with either BCG of the Pasteur strain (BCG-P), BCG of the Connought strain (BCG-C), 1 mg of heat-killed *M. vaccae*, or 200 μg of DD-*M. vaccae* prior to challenge with ovalbumin.

Fig. 3A illustrates the effect of immunizing mice with heat-killed *M. vaccae* or delipidated and deglycolipidated *M. vaccae* (DD-*M. vaccae*) prior to infection with tuberculosis. Fig. 3B illustrates the effect of immunizing mice with heat-killed *M. vaccae*, recombinant *M. vaccae* proteins, or a combination of heat-killed *M. vaccae* and *M. vaccae* recombinant proteins prior to infection with tuberculosis.

Fig. 4 illustrates the induction of IL-12 by autoclaved *M. vaccae*, lyophilized *M. vaccae*, delipidated and deglycolipidated *M. vaccae* and *M. vaccae* glycolipids.

Fig. 5 compares the *in vitro* stimulation of interferon-gamma production in spleen cells from Severe Combined ImmunoDeficient (SCID) mice by different concentrations of heat-killed (autoclaved) *M. vaccae*, delipidated and deglycolipidated *M. vaccae*, and *M. vaccae* glycolipids.

Figs. 6A, B and C illustrate the stimulation of interferon-gamma production by different concentrations of *M. vaccae* recombinant proteins, heat-killed *M. vaccae*, delipidated and deglycolipidated *M. vaccae* (referred to in the figure as "delipidated *M. vaccae*"), *M. vaccae* glycolipids and lipopolysaccharide, in peritoneal macrophages from C57BL/6 mice (Fig. 6A), BALB/C mice (Fig. 6B) or C3H/HeJ mice (Fig. 6C).

Fig. 7A(i) - (iv) illustrate the non-specific immune amplifying effects of 10 μg, 100 μg and 1mg autoclaved *M. vaccae* and 75 μg unfractionated culture filtrates of *M. vaccae,* respectively. Fig. 7B(i) and (ii) illustrate the non-specific immune amplifying effects of autoclaved *M. vaccae,* and delipidated and deglycolipidated *M. vaccae*, respectively. Fig. 7C(i) illustrates the non-specific immune amplifying effects of whole autoclaved *M. vaccae*. Fig. 7C(ii) illustrates the non-specific immune amplifying effects of soluble *M. vaccae* proteins extracted with SDS from delipidated and deglycolipidated *M. vaccae*. Fig. 7C(iii) illustrates that the non-specific amplifying effects of the preparation of Fig. 7C(ii) are destroyed by treatment with the proteolytic enzyme Pronase. Fig. 7D illustrates the non-specific immune amplifying effects of heat-killed *M. vaccae* (Fig. 7D(i)), whereas a non-specific immune amplifying effect was not seen with heat-killed preparations of *M. tuberculosis* (Fig. 7D(ii)), *M. bovis* BCG (Fig. 7D(iii)), *M. phlei* (Fig. 7D(iv)) and *M. smegmatis* (Fig. 7D(v)).

Figs. 8A and B illustrate the stimulation of CD69 expression on αβT cells, γδT cells and NK cells, respectively, by the *M. vaccae* protein GV23, the Th1-inducing adjuvants MPL/TDM/CWS and CpG ODN, and the Th2-inducing adjuvants aluminium hydroxide and cholera toxin.

Figs. 9A-D illustrate the effect of heat-killed *M. vaccae*, DD-*M. vaccae* and *M. vaccae* recombinant proteins on the production of IL-1β, TNF-α, IL-12 and IFN-γ, respectively, by human PBMC.

Figs. 10A-C illustrate the effects of varying concentrations of the recombinant *M. vaccae* proteins GV-23 and GV-45 on the production of IL-1β, TNF-α and IL-12, respectively, by human PBMC.

Figs. 11A-D illustrate the stimulation of IL-1β, TNF-α, IL-12 and IFN-γ production, respectively, in human PBMC by the *M. vaccae* protein GV23, the Th1-inducing adjuvants MPL/TDM/CWS and CpG ODN, and the Th2-inducing adjuvants aluminium hydroxide and cholera toxin.

Figs. 12A-C illustrate the effects of varying concentrations of the recombinant *M. vaccae* proteins GV-23 and GV-45 on the expression of CD40, CD80 and CD86, respectively, by dendritic cells.

Fig. 13 illustrates the enhancement of dendritic cell mixed leukocyte reaction by the recombinant *M. vaccae* protein GV-23.

Detailed Description of the Invention

As noted above, the present invention is generally directed to compositions and methods for preventing, treating and diagnosing infectious diseases and immune disorders. Disorders which may be effectively treated using the inventive compositions include diseases of the respiratory system, such as mycobacterial infections, asthma, sarcoidosis and lung cancers, and disorders of the skin, such as psoriasis, atopic dermatis, allergic contact dermatitis, alopecia areata, and the skin cancers basal cell carcinoma, squamous cell carcinoma and melanoma.

Effective vaccines that provide protection against infectious microorganisms contain at least two functionally different components. The first is an antigen, which may be polypeptide or carbohydrate in nature, and which is processed by macrophages and other antigen-presenting cells and displayed for $CD4^+$ T cells or for $CD8^+$ T cells. This antigen forms the "specific" target of an immune response. The second component of a vaccine is a non-specific immune response amplifier, termed an adjuvant, with which the antigen is mixed or is incorporated into. An adjuvant amplifies either cell-mediated or antibody immune responses to a structurally unrelated compound or polypeptide. Several known adjuvants are prepared from microbes such as *Bordetella pertussis*, *M. tuberculosis* and *M. bovis* BCG. Adjuvants may also contain components designed to protect polypeptide antigens from degradation, such as aluminum hydroxide or mineral oil. While the antigenic component of a vaccine contains polypeptides that direct the immune attack against a specific pathogen, such as *M. tuberculosis*, the adjuvant is often capable of broad use in many different vaccine formulations. Certain known proteins, such as bacterial enterotoxins, can function both as an

antigen to elicit a specific immune response and as an adjuvant to enhance immune responses to unrelated proteins.

Certain pathogens, such as *M. tuberculosis*, as well as certain cancers, are effectively contained by an immune attack directed by $CD4^+$ and $CD8^+$ T cells, known as cell-mediated immunity. Other pathogens, such as poliovirus, also require antibodies, produced by B cells, for containment. These different classes of immune attack (T cell or B cell) are controlled by different subpopulations of $CD4^+$ T cells, commonly referred to as Th1 and Th2 cells. A desirable property of an adjuvant is the ability to selectively amplify the function of either Th1 or Th2 populations of $CD4^+$ T cells. Many skin disorders, including psoriasis, atopic dermatitis, alopecia, and skin cancers appear to be influenced by differences in the activity of these Th cell subsets.

The two types of Th cell subsets have been well characterized in a murine model and are defined by the cytokines they release upon activation. The Th1 subset secretes IL-2, IFN-γ and tumor necrosis factor, and mediates macrophage activation and delayed-type hypersensitivity response. The Th2 subset releases IL-4, IL-5, IL-6 and IL-10, which stimulate B cell activation. The Th1 and Th2 subsets are mutually inhibiting, so that IL-4 inhibits Th1-type responses, and IFN-γ inhibits Th2-type responses. Similar Th1 and Th2 subsets have been found in humans, with release of the identical cytokines observed in the murine model. In particular, the majority of T-cell clones from atopic human lymphocytes resemble the murine Th2 cell that produces IL-4, whereas very few clones produce IFN-γ. Therefore, the selective expression of the Th2 subset with subsequent production of IL-4 and decreased levels of IFN-γ-producing cells could lead to preferential enhancement of IgE production. Amplification of Th1-type immune responses is central to a reversal of disease state in many disorders, including disorders of the respiratory system such as tuberculosis, sarcoidosis, asthma, allergic rhinitis and lung cancers.

Inactivated *M. vaccae* and many compounds derived from *M. vaccae* have both antigen and adjuvant properties which function to enhance Th1-type immune responses. The methods of the present invention employ one or more of these antigen and adjuvant compounds from *M. vaccae* and/or its culture filtrates to redirect immune activities of T cells

in patients. Mixtures of such compounds are particularly effective in the methods disclosed herein. While it is well known that all mycobacteria contain many cross-reacting antigens, it is not known whether they contain adjuvant compounds in common. As shown below, inactivated *M. vaccae* and a modified (delipidated and deglycolipidated) form of inactivated *M. vaccae* have been found to have adjuvant properties of the Th1-type which are not shared by a number of other mycobacterial species. Furthermore, it has been found that *M. vaccae* produces compounds in its own culture filtrate which amplify the immune response to *M. vaccae* antigens also found in culture filtrate, as well as to antigens from other sources.

In one aspect, the present invention provides methods for the immunotherapy of respiratory and/or lung disorders, including tuberculosis, sarcoidosis, asthma, allergic rhinitis and lung cancers, in a patient to enhance Th1-type immune responses. In one embodiment, the compositions are delivered directly to the mucosal surfaces of airways leading to and/or within the lungs. However, the compositions may also be administered via intradermal or subcutaneous routes. Compositions which may be usefully employed in such methods comprise at least one of the following components: inactivated *M. vaccae* cells; *M. vaccae* culture filtrate; delipidated and deglycolipidated *M. vaccae* cells (DD-*M. vaccae*); and compounds present in or derived from *M. vaccae* and/or its culture filtrate. As illustrated below, administration of such compositions, results in specific T cell immune responses and enhanced protection against *M. tuberculosis* infection, and is also effective in the treatment of asthma. While the precise mode of action of these compositions in the treatment of diseases such as asthma is unknown, they are believed to suppress an asthma-inducing Th2 immune response.

As used herein the term "respiratory system" refers to the lungs, nasal passageways, trachea and bronchial passageways.

As used herein the term "airways leading to or located in the lung" includes the nasal passageways, mouth, tonsil tissue, trachea and bronchial passageways.

As used herein, a "patient" refers to any warm-blooded animal, preferably a human. Such a patient may be afflicted with disease or may be free of detectable disease. In other

words, the inventive methods may be employed to induce protective immunity for the prevention or treatment of disease.

In another aspect, the present invention provides methods for the immunotherapy of skin disorders, including psoriasis, atopic dermatitis, alopecia, and skin cancers in patients, in which immunotherapeutic agents are employed to alter or redirect an existing state of immune activity by altering the function of T cells to a Th1-type of immune response. Compositions which may be usefully employed in the inventive methods comprise at least one of the following components: inactivated *M. vaccae* cells; *M. vaccae* culture filtrate; modified *M. vaccae* cells; and constituents and compounds present in or derived from *M. vaccae* and/or its culture filtrate. As detailed below, multiple administrations of such compositions, preferably by intradermal injection, have been shown to be highly effective in the treatment of psoriasis.

As used herein the term "inactivated *M. vaccae*" refers to *M. vaccae* that have either been killed by means of heat, as detailed below in Example 7, or subjected to radiation, such as ^{60}Cobalt at a dose of 2.5 megarads. As used herein, the term "modified *M. vaccae*" includes delipidated *M. vaccae* cells, deglycolipidated *M. vaccae* cells and *M. vaccae* cells that have been both delipidated and deglycolipidated (DD-*M. vaccae*).

The preparation of DD-*M. vaccae* and its chemical composition are described below in Example 7. As detailed below, the inventors have shown that removal of the glycolipid constituents from *M. vaccae* results in the removal of molecular components that stimulate interferon-gamma production in natural killer (NK) cells, thereby significantly reducing the non-specific production of a cytokine that has numerous harmful side-effects.

In yet a further aspect, the present invention provides isolated polypeptides that comprise at least one immunogenic portion of a *M. vaccae* antigen, or a variant thereof, or at least one adjuvant porition of an M. vaccae protein. In specific embodiments, such polypeptides comprise an immunogenic portion of an antigen, or a variant thereof, wherein the antigen includes a sequence selected from the group consisting of SEQ ID NO: 1-4, 9-16, 18-21, 23, 25, 26, 28, 29, 44, 45, 47, 52-55, 63, 64, 70, 75, 89, 94, 98, 100-105, 109, 110, 112, 121, 124, 125, 134, 135, 140, 141, 143, 145, 147, 152, 154, 156, 158, 160, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 201, 203, 205 and 207.

As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins (i.e., antigens), wherein the amino acid residues are linked by covalent peptide bonds. Thus, a polypeptide comprising an immunogenic portion of one of the above antigens may consist entirely of the immunogenic portion, or may contain additional sequences. The additional sequences may be derived from the native *M. vaccae* antigen or may be heterologous, and such sequences may (but need not) be immunogenic. As detailed below, polypeptides of the present invention may be isolated from *M. vaccae* cells or culture filtrate, or may be prepared by synthetic or recombinant means.

"Immunogenic," as used herein, refers to the ability to elicit an immune response in a patient, such as a human, or in a biological sample. In particular, immunogenic antigens are capable of stimulating cell proliferation, interleukin-12 production or interferon-γ production in biological samples comprising one or more cells selected from the group of T cells, NK cells, B cells and macrophages, where the cells are derived from an *M. tuberculosis*-immune individual. Exposure to an immunogenic antigen generally results in the generation of immune memory such that upon re-exposure to that antigen, an enhanced and more rapid response occurs.

Immunogenic portions of the antigens described herein may be prepared and identified using well known techniques, such as those summarised in Paul, *Fundamental Immunology,* 3d ed., Raven Press, 1993, pp. 243-247. Such techniques include screening polypeptide portions of the native antigen or protein for immunogenic properties. The representative proliferation and cytokine production assays described herein may be employed in these screens. An immunogenic portion of an antigen is a portion that, within such representative assays, generates an immune response (*e.g.,* cell proliferation, interferon-γ production or interleukin-12 production) that is substantially similar to that generated by the full-length antigen. In other words, an immunogenic portion of an antigen may generate at least about 20%, preferably about 65%, and most preferably about 100% of the proliferation induced by the full-length antigen in the model proliferation assay described herein. An immunogenic portion may also, or alternatively, stimulate the production of at least about 20%, preferably

about 65% and most preferably about 100%, of the interferon-γ and/or interleukin-12 induced by the full length antigen in the model assay described herein.

A *M. vaccae* adjuvant is a compound found in *M. vaccae* cells or *M. vaccae* culture filtrates which non-specifically stimulates immune responses. Adjuvants enhance the immune response to immunogenic antigens and the process of memory formation. In the case of *M. vaccae* proteins, these memory responses favour Th1-type immunity. Adjuvants are also capable of stimulating interleukin-12 production or interferon-γ production in biological samples comprising one or more cells selected from the group of T cells, NK cells, B cells and macrophages, where the cells are derived from healthy individuals. Adjuvants may or may not stimulate cell proliferation. Such *M. vaccae* adjuvants include, for example, polypeptides comprising a sequence recited in SEQ ID NO: 89, 117, 160, 162 or 201.

The term "polynucleotide(s)," as used herein, means a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including HnRNA and mRNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An HnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An mRNA molecule corresponds to an HnRNA and DNA molecule from which the introns have been excised. A polynucleotide may consist of an entire gene, or any portion thereof. Operable anti-sense polynucleotides may comprise a fragment of the corresponding polynucleotide, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments.

The compositions and methods of this invention also encompass variants of the above polypeptides and polynucleotides. As used herein, the term "variant" covers any sequence which has at least about 40%, more preferably at least about 60%, more preferably yet at least about 75% and most preferably at least about 90% identical residues (either nucleotides or amino acids) to a sequence of the present invention. The percentage of identical residues is determined by aligning the two sequences to be compared, determining the number of identical residues in the aligned portion, dividing that number by the total length of the inventive, or queried, sequence and multiplying the result by 100.

Polynucleotide or polypeptide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another polynucleotide, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. Both the BLASTN and BLASTP software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN and BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F., et al. (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res. 25*:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu/pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in W.R. Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA 85*:2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology 183*:63-98 (1990).

The following running parameters are preferred for determination of alignments and similarities using BLASTN that contribute to the E values and percentage identity: Unix running command: blastall -p blastn -d embldb -e 10 -G 1 -E 1 -r 2 -v 50 -b 50 -i queryseq – o results; and parameter default values:

-p Program Name [String]

-d Database [String]

-e Expectation value (E) [Real]

-G Cost to open a gap (zero invokes default behavior) [Integer]

-E Cost to extend a gap (zero invokes default behavior) [Integer]

-r Reward for a nucleotide match (blastn only) [Integer]

-v Number of one-line descriptions (V) [Integer]

-b Number of alignments to show (B) [Integer]

-i Query File [File In]

-o BLAST report Output File [File Out] Optional

For BLASTP the following running parameters are preferred: blastall –p blastp –d swissprotdb –e 10 –G 1 –E 1 –v 50 –b 50 –i queryseq –o results

-p Program Name [String]

-d Database [String]

-e Expectation value (E) [Real]

-G Cost to open a gap (zero invokes default behavior) [Integer]

-E Cost to extend a gap (zero invokes default behavior) [Integer]

-v Number of one-line descriptions (v) [Integer]

-b Number of alignments to show (b) [Integer]

-I Query File [File In]

-o BLAST report Output File [File Out] Optional

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, BLASTP, FASTA, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN and FASTA algorithms also produce "Expect" values for alignments. The Expect value (E) indicates the number of hits one can "expect" to see over a certain number of contiguous sequences by chance when searching a database of a certain size. The Expect value is used as a significance threshold for determining whether the hit to a database, such as the preferred EMBL database, indicates true similarity. For example, an E value of 0.1 assigned to a hit is interpreted as meaning that in a database of the size of the EMBL database, one might expect to see 0.1 matches over the aligned portion of the sequence with a

similar score simply by chance. By this criterion, the aligned and matched portions of the sequences then have a probability of 90% of being the same. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in the EMBL database is 1% or less using the BLASTN or FASTA algorithm.

According to one embodiment, "variant" polynucleotides, with reference to each of the polynucleotides of the present invention, preferably comprise sequences having the same number or fewer nucleic acids than each of the polynucleotides of the present invention and producing an E value of 0.01 or less when compared to the polynucleotide of the present invention. That is, a variant polynucleotide is any sequence that has at least a 99% probability of being the same as the polynucleotide of the present invention, measured as having an E value of 0.01 or less using the BLASTN or FASTA algorithms set at the default parameters. According to a preferred embodiment, a variant polynucleotide is a sequence having the same number or fewer nucleic acids than a polynucleotide of the present invention that has at least a 99% probability of being the same as the polynucleotide of the present invention, measured as having an E value of 0.01 or less using the BLASTN or FASTA algorithms set at the default parameters.

Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65 °C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65 °C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65 °C.

Portions and other variants of *M. vaccae* polypeptides may be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied

BioSystems, Inc. (Foster City, CA), and may be operated according to the manufacturer's instructions. Variants of a native antigen or adjuvant may be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site specific mutagenesis. Sections of the DNA sequence may also be removed using standard techniques to permit preparation of truncated polypeptides.

A polypeptide of the present invention may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

In general, *M. vaccae* antigens, and DNA sequences encoding such antigens, may be prepared using any of a variety of procedures. For example, soluble antigens may be isolated from *M. vaccae* culture filtrate as described below. Antigens may also be produced recombinantly by inserting a DNA sequence that encodes the antigen into an expression vector and expressing the antigen in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, mycobacteria, insect, yeast or a mammalian cell line such as COS or CHO. The DNA sequences expressed in this manner may encode naturally occurring antigens, portions of naturally occurring antigens, or other variants thereof.

DNA sequences encoding *M. vaccae* antigens may be obtained by screening an appropriate *M. vaccae* cDNA or genomic DNA library for DNA sequences that hybridize to degenerate oligonucleotides derived from partial amino acid sequences of isolated soluble antigens. Suitable degenerate oligonucleotides may be designed and synthesized, and the screen may be performed as described, for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989. As

described below, polymerase chain reaction (PCR) may be employed to isolate a nucleic acid probe from genomic DNA, or a cDNA or genomic DNA library. The library screen may then be performed using the isolated probe. DNA molecules encoding *M. vaccae* antigens may also be isolated by screening an appropriate *M. vaccae* expression library with anti-sera (*e.g.*, rabbit or monkey) raised specifically against *M. vaccae* antigens.

Regardless of the method of preparation, the antigens described herein have the ability to induce an immunogenic response. More specifically, the antigens have the ability to induce cell proliferation and/or cytokine production (for example, interferon-γ and/or interleukin-12 production) in T cells, NK cells, B cells or macrophages derived from an *M. tuberculosis*-immune individual. An *M. tuberculosis*-immune individual is one who is considered to be resistant to the development of tuberculosis by virtue of having mounted an effective T cell response to *M. tuberculosis*. Such individuals may be identified based on a strongly positive (*i.e.*, greater than about 10 mm diameter induration) intradermal skin test response to tuberculosis proteins (PPD), and an absence of any symptoms of tuberculosis infection.

Assays for cell proliferation or cytokine production in T cells, NK cells, B cells or macrophages may be performed, for example, using the procedures described below. The selection of cell type for use in evaluating an immunogenic response to an antigen will depend on the desired response. For example, interleukin-12 production is most readily evaluated using preparations containing T cells, NK cells, B cells and macrophages derived from *M. tuberculosis*-immune individuals may be prepared using methods well known in the art. For example, a preparation of peripheral blood mononuclear cells (PBMCs) may be employed without further separation of component cells. PBMCs may be prepared, for example, using density centrifugation through Ficoll™ (Winthrop Laboratories, NY). T cells for use in the assays described herein may be purified directly from PBMCs. Alternatively, an enriched T cell line reactive against mycobacterial proteins, or T cell clones reactive to individual mycobacterial proteins, may be employed. Such T cell clones may be generated by, for example, culturing PBMCs from *M. tuberculosis*-immune individuals with mycobacterial proteins for a period of 2-4 weeks. This allows expansion of only the mycobacterial protein-specific T cells, resulting in a line composed solely of such cells. These cells may then be

cloned and tested with individual proteins, using methods well known in the art, to more accurately define individual T cell specificity.

In general, regardless of the method of preparation, the polypeptides disclosed herein are prepared in an isolated, substantially pure, form. Preferably, the polypeptides are at least about 80% pure, more preferably at least about 90% pure and most preferably at least about 99% pure. In certain preferred embodiments, described in detail below, the substantially pure polypeptides are incorporated into pharmaceutical compositions or vaccines for use in one or more of the methods disclosed herein.

The present invention also provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, a polypeptide of the present invention and a known *M tuberculosis* antigen, such as the 38 kDa antigen described in Andersen and Hansen, *Infect. Immun.* 57:2481-2488, 1989, together with variants of such fusion proteins. The fusion proteins of the present invention may also include a linker peptide between the first and second polypeptides.

A DNA sequence encoding a fusion protein of the present invention is constructed using known recombinant DNA techniques to assemble separate DNA sequences encoding the first and second polypeptides into an appropriate expression vector. The 3' end of a DNA sequence encoding the first polypeptide is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide so that the reading frames of the sequences are in phase to permit mRNA translation of the two DNA sequences into a single fusion protein that retains the biological activity of both the first and the second polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptides by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide

linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may be from 1 to about 50 amino acids in length. Peptide linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference. The ligated DNA sequences encoding the fusion proteins are cloned into suitable expression systems using techniques known to those of ordinary skill in the art.

As detailed below, the inventors have demonstrated that heat-killed *M. vaccae*, DD-*M. vaccae* and recombinant *M. vaccae* proteins of the present invention may be employed to activate T cells and NK cells; to stimulate the production of cytokines (in particular Th1 class of cytokines) in human PBMC; to enhance the expression of co-stimulatory molecules on dendritic cells and monocytes (thereby enhancing activation); and to enhance dendritic cell maturation and function. Furthermore, the inventors have demonstrated similarities between the immunological properties of the inventive *M. vaccae* protein GV-23 and those of two known Th1-inducing adjuvants. GV-23 may thus be employed in the treatment of diseases that involve enhancing a Th1 immune response. Examples of such diseases include allergic diseases (for example, asthma and eczema) autoimmune diseases (for example, systemic lupus erythematosus) and infectious diseases (for example, tuberculosis and leprosy). In addition, GV-23 may be employed as a dendritic cell or NK cell enhancer in the treatment of immune deficiency disorders, such as HIV, and to enhance immune responses and cytotoxic responses to, for example, malignant cells in cancer and following immunosuppressive anti-cancer therapies, such as chemotherapy.

For use in the inventive therapeutic methods, the inactivated *M. vaccae*, *M. vaccae* culture filtrate, modified *M. vaccae* cells, *M. vaccae* polypeptide, fusion protein (or polynucleotides encoding such polypeptides or fusion proteins) is generally present within a pharmaceutical composition or a vaccine. Pharmaceutical compositions may comprise one or

more components selected from the group consisting of inactivated *M. vaccae* cells, *M. vaccae* culture filtrate, modified *M. vaccae* cells, and compounds present in or derived from *M. vaccae* and/or its culture filtrate, together with a physiologically acceptable carrier. Vaccines may comprise one or more components selected from the group consisting of inactivated *M. vaccae* cells, *M. vaccae* culture filtrate, modified *M. vaccae* cells, and compounds present in or derived from *M. vaccae* and/or its culture filtrate, together with a non-specific immune response amplifier. Such pharmaceutical compositions and vaccines may also contain other mycobacterial antigens, either, as discussed above, incorporated into a fusion protein or present within a separate polypeptide.

Alternatively, a vaccine of the present invention may contain DNA encoding one or more polypeptides as described above, such that the polypeptide is generated *in situ*. In such vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacterial and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminator signal). Bacterial delivery systems involve the administration of a bacterium (such as Bacillus-Calmette-Guerin) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other poxvirus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic, or defective, replication competent virus. Techniques for incorporating DNA into such expression systems are well known in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

A DNA vaccine as described above may be administered simultaneously with or sequentially to either a polypeptide of the present invention or a known mycobacterial antigen, such as the 38 kDa antigen described above. For example, administration of DNA encoding a polypeptide of the present invention, may be followed by administration of an antigen in order to enhance the protective immune effect of the vaccine.

Routes and frequency of administration, as well as dosage, will vary from individual to individual and may parallel those currently being used in immunization using BCG. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intradermal, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Between 1 and 3 doses may be administered for a 1-36 week period. Preferably, 3 doses are administered, at intervals of 3-4 months, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of polypeptide or DNA that, when administered as described above, is capable of raising an immune response in a patient sufficient to protect the patient from mycobacterial infection for at least 1-2 years. In general, the amount of polypeptide present in a dose (or produced *in situ* by the DNA in a dose) ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to about 1 mg, and preferably from about 100 pg to about 1 μg. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 ml to about 5 ml.

In one embodiment, the pharmaceutical composition or vaccine is in a form suitable for delivery to the mucosal surfaces of the airways leading to or within the lungs. For example, the pharmaceutical composition or vaccine may be suspended in a liquid formulation for delivery to a patient in an aerosol form or by means of a nebulizer device similar to those currently employed in the treatment of asthma. In other embodiments, the pharmaceutical composition or vaccine is in a form suitable for administration by injection (intracutaneous, intramuscular, intravenous or subcutaneous) or orally. While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will depend on the suitability for the chosen route of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a lipid, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable

biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Any of a variety of adjuvants may be employed in the vaccines of this invention to non-specifically enhance the immune response. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a non-specific stimulator of immune responses, such as lipid A, *Bordetella pertussis*, *M. tuberculosis*, or, as discussed below, *M. vaccae*. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI), and Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ). Other suitable adjuvants include alum, biodegradable microspheres, monophosphoryl lipid A and Quil A.

In another aspect, this invention provides methods for using one or more of the inventive polypeptides to diagnose tuberculosis using a skin test. As used herein, a "skin test" is any assay performed directly on a patient in which a delayed-type hypersensitivity (DTH) reaction (such as swelling, reddening or dermatitis) is measured following intradermal injection of one or more polypeptides as described above. Preferably, the reaction is measured at least 48 hours after injection, more preferably 48-72 hours.

The DTH reaction is a cell-mediated immune response, which is greater in patients that have been exposed previously to the test antigen (*i.e.*, the immunogenic portion of the polypeptide employed, or a variant thereof). The response may be measured visually, using a ruler. In general, a response that is greater than about 0.5 cm in diameter, preferably greater than about 1.0 cm in diameter, is a positive response, indicative of tuberculosis infection.

For use in a skin test, the polypeptides of the present invention are preferably formulated, as pharmaceutical compositions containing a polypeptide and a physiologically acceptable carrier, as described above. Such compositions typically contain one or more of the above polypeptides in an amount ranging from about 1 μg to about 100 μg, preferably from about 10 μg to about 50 μg in a volume of 0.1 ml. Preferably, the carrier employed in such pharmaceutical compositions is a saline solution with appropriate preservatives, such as phenol and/or Tween 80™.

In a preferred embodiment, a polypeptide employed in a skin test is of sufficient size such that it remains at the site of injection for the duration of the reaction period. In general, a polypeptide that is at least 9 amino acids in length is sufficient. The polypeptide is also preferably broken down by macrophages or dendritic cells within hours of injection to allow presentation to T-cells. Such polypeptides may contain repeats of one or more of the above sequences or other immunogenic or nonimmunogenic sequences.

In another aspect, methods are provided for detecting mycobacterial infection in a biological sample, using one or more of the inventive polypeptides, either alone or in combination. In embodiments in which multiple polypeptides are employed, polypeptides other than those specifically described herein, such as the 38 kDa antigen described above, may be included. As used herein, a "biological sample" is any antibody-containing sample obtained from a patient. Preferably, the sample is whole blood, sputum, serum, plasma, saliva, cerebrospinal fluid or urine. More preferably, the sample is a blood, serum or plasma sample obtained from a patient or a blood supply. The polypeptide(s) are used in an assay, as described below, to determine the presence or absence of antibodies to the polypeptide(s) in the sample, relative to a predetermined cut-off value. The presence of such antibodies indicates the presence of mycobacterial infection.

In embodiments in which more than one polypeptide is employed, the polypeptides used are preferably complementary (*i.e.*, one component polypeptide will tend to detect infection in samples where the infection would not be detected by another component polypeptide). Complementary polypeptides may generally be identified by using each polypeptide individually to evaluate serum samples obtained from a series of patients known to be infected with a *Mycobacterium*. After determining which samples test positive (as described below) with each polypeptide, combinations of two or more polypeptides may be formulated that are capable of detecting infection in most, or all, of the samples tested. For example, approximately 25-30% of sera from tuberculosis-infected individuals are negative for antibodies to any single protein, such as the 38 kDa antigen mentioned above. Complementary polypeptides may, therefore, be used in combination with the 38 kDa antigen to improve sensitivity of a diagnostic test.

A variety of assay formats employing one or more polypeptides to detect antibodies in a sample are well known in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In a preferred embodiment, the assay involves the use of polypeptide immobilized on a solid support to bind to and remove the antibody from the sample. The bound antibody may then be detected using a detection reagent that contains a reporter group. Suitable detection reagents include antibodies that bind to the antibody/polypeptide complex and free polypeptide labelled with a reporter group (*e.g.*, in a semi-competitive assay). Alternatively, a competitive assay may be utilized, in which an antibody that binds to the polypeptide is labelled with a reporter group and allowed to bind to the immobilized antigen after incubation of the antigen with the sample. The extent to which components of the sample inhibit the binding of the labelled antibody to the polypeptide is indicative of the reactivity of the sample with the immobilized polypeptide.

The solid support may be any solid material to which the antigen may be attached. Suitable materials are well known in the art. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681.

The polypeptides may be bound to the solid support using a variety of techniques well known in the art. In the context of the present invention, the term "bound" refers to both noncovalent association, such as adsorption, and covalent attachment, which may be a direct linkage between the antigen and functional groups on the support or a linkage by way of a cross-linking agent. Binding by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the polypeptide, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of polypeptide ranging from about 10 ng to about 1 μg, and preferably about 100 ng, is sufficient to bind an adequate amount of antigen.

Covalent attachment of polypeptide to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the polypeptide. For example, the polypeptide may be bound to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the polypeptide (*see*, *e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is an enzyme-linked immunosorbent assay (ELISA). This assay may be performed by first contacting a polypeptide antigen that has been immobilized on a solid support, with the sample, such that antibodies to the polypeptide within the sample are allowed to bind to the immobilized polypeptide. Unbound sample is then removed from the immobilized polypeptide and a detection reagent capable of binding to the immobilized antibody-polypeptide complex is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific detection reagent.

More specifically, once the polypeptide is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO) may be employed. The immobilized polypeptide is then incubated with the sample, and antibody is allowed to bind to the antigen. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time, or incubation time, is that period of time that is sufficient to detect the presence of antibody within a *M. tuberculosis*-infected sample. Preferably, the contact time is sufficient to achieve a level of binding that is at least 95% of that achieved at equilibrium between bound and unbound antibody. The time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. Detection reagent may then be added to the solid support. An appropriate detection reagent is any compound that binds to the immobilized antibody-polypeptide complex and that can be detected by any of a variety of means known in the art. Preferably, the detection reagent contains a binding agent (such as, for example, Protein A, Protein G, immunoglobulin, lectin or free antigen) conjugated to a reporter group. Preferred reporter groups include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. The conjugation of binding agent to reporter group may be achieved using standard methods known in the art. Common binding agents may also be purchased conjugated to a variety of reporter groups from many commercial sources (*e.g.*, Zymed Laboratories, San Francisco, CA, and Pierce, Rockford, IL).

The detection reagent is incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound antibody. An appropriate amount of time may generally be determined from the manufacturer's instructions or by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of anti-mycobacterial antibodies in the sample, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value is the average mean signal obtained when the immobilized antigen is incubated with samples from an uninfected patient. In an alternate

preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, pp. 106-107. In general, signals higher than the predetermined cut-off value are considered to be positive for mycobacterial infection.

The assay may also be performed in a rapid flow-through or strip test format, wherein the antigen is immobilized on a membrane, such as nitrocellulose. In the flow-through test, antibodies within the sample bind to the immobilized polypeptide as the sample passes through the membrane. A detection reagent (*e.g.*, protein A-colloidal gold) then binds to the antibody-polypeptide complex as the solution containing the detection reagent flows through the membrane. The detection of bound detection reagent may then be performed as described above. In the strip test format, one end of the membrane to which polypeptide is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing detection reagent and to the area of immobilized polypeptide. Concentration of detection reagent at the polypeptide indicates the presence of anti-mycobacterial antibodies in the sample. Typically, the concentration of detection reagent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of polypeptide immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of antibodies that would be sufficient to generate a positive signal in an ELISA, as discussed above. Preferably, the amount of polypeptide immobilized on the membrane ranges from about 25 ng to about 1 μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount (*e.g.*, one drop) of patient serum or blood.

Numerous other assay protocols exist that are suitable for use with the polypeptides of the present invention. The above descriptions are intended to be exemplary only.

The present invention also provides antibodies to the inventive polypeptides. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In one such technique, an immunogen comprising the antigenic

polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep and goats). The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* *6*:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells may then be immortalized by fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal, using one of a variety of techniques well known in the art.

Monoclonal antibodies may be isolated from the supernatants of the resulting hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood.

Antibodies may be used in diagnostic tests to detect the presence of mycobacterial antigens using assays similar to those detailed above and other techniques well known to those of skill in the art, thereby providing a method for detecting mycobacterial infection, such as *M. tuberculosis* infection, in a patient.

Diagnostic reagents of the present invention may also comprise polynucleotides encoding one or more of the above polypeptides, or one or more portions thereof. For example, primers comprising at least 10 contiguous oligonucleotides of an inventive polynucleotide may be used in polymerase chain reaction (PCR) based tests. Similarly, probes comprising at least 18 contiguous oligonucleotides of an inventive polynucleotide may

be used for hybridizing to specific sequences. Techniques for both PCR based tests and hybridization tests are well known in the art. Primers or probes may thus be used to detect *M. tuberculosis* and other mycobacterial infections in biological samples, preferably sputum, blood, serum, saliva, cerebrospinal fluid or urine. DNA probes or primers comprising oligonucleotide sequences described above may be used alone, in combination with each other, or with previously identified sequences, such as the 38 kDa antigen discussed above.

The word "about," when used in this application with reference to a percentage by weight composition, contemplates a variance of up to 10 percentage units from the stated percentage. When used in reference to percentage identity or percentage probability, the word "about" contemplates a variance of up to one percentage unit from the stated percentage.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

EFFECT OF IMMUNIZATION OF MICE WITH *M. VACCAE* ON TUBERCULOSIS

This example illustrates the effect of immunization with heat-killed *M. vaccae* or *M. vaccae* culture filtrate in mice prior to challenge with live *M. tuberculosis*.

M. vaccae (ATCC Number 15483) was cultured in sterile Medium 90 (yeast extract, 2.5 g/l; tryptone, 5 g/l; glucose, 1 g/l) at 37 °C. The cells were harvested by centrifugation, and transferred into sterile Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) with glucose at 37 °C for one day. The medium was then centrifuged to pellet the bacteria, and the culture filtrate removed. The bacterial pellet was resuspended in phosphate buffered saline at a concentration of 10 mg/ml, equivalent to 10^{10} *M. vaccae* organisms per ml. The cell suspension was then autoclaved for 15 min at 120 °C. The culture filtrate was passaged through a 0.45 μm filter into sterile bottles.

As shown in Fig.1A, when mice were immunized with 1 mg, 100 μg or 10 μg of *M. vaccae* and infected three weeks later with $5x10^5$ colony forming units (CFU) of live *M. tuberculosis* H37Rv, significant protection from infection was seen. In this example, spleen,

liver and lung tissue was harvested from mice three weeks after infection, and live bacilli determined (expressed as CFU). The reduction in bacilli numbers, when compared to tissue from non-immunized control mice, exceeded 2 logs in liver and lung tissue, and 1 log in spleen tissue. Immunization of mice with heat-killed *M. tuberculosis* H37Rv had no significant protective effects on mice subsequently infected with live *M. tuberculosis* H37Rv.

Fig.1B shows that when mice were immunized with 100 μg of *M. vaccae* culture filtrate, and infected three weeks later with $5x10^5$ CFU of *M. tuberculosis* H37Rv, significant protection was also seen. When spleen, liver and lung tissue was harvested from mice three weeks after infection, and live bacilli numbers (CFU) determined, a 1-2 log reduction in numbers, as compared to non-immunized control mice, was observed.

EXAMPLE 2

EFFECT OF INTRADERMAL AND INTRA-LUNG ROUTES OF IMMUNISATION WITH *M. VACCAE* ON TUBERCULOSIS IN CYNOMOLGOUS MONKEYS

This example illustrates the effect of immunisation with heat-killed *M. vaccae* or *M. vaccae* culture filtrate through intradermal and intralung routes in cynomolgous monkeys prior to challenge with live *M. tuberculosis*.

Heat-killed *M. vaccae* and *M. vaccae* culture filtrate were prepared as described above in Example 1. Five groups of cynomolgous monkeys were used, with each group containing 2 monkeys. Two groups of monkeys were immunised with whole heat-killed *M. vaccae* either intradermally or intralung; two groups of monkeys were immunised with *M. vaccae* culture filtrate either intradermally or intralung; and a control group received no immunisations. All immunogens were dissolved in phosphate buffered saline. The composition employed for immunisation, amount of immunogen, and route of administration for each group of monkeys are provided in Table 1. Prior to immunisation, all monkeys were weighed (Wt kg), body temperature was measured (temp), and a blood sample taken for determination of erythrocyte sedimentation rate (ESR mm/hr) and lymphocyte proliferation (LPA) to an *in vitro* challenge

with purified protein (PPD) prepared from *Mycobacterium bovis*. Both ESR and LPA have been used as indicators of inflammatory T cell responses. At day 33 post-immunisation these measurements were repeated. At day 34, all monkeys received a second immunisation using the same amount of *M. vaccae* and route of immunisation as the initial immunisation. On day 62, body weight, temperature, ESR and LPA to PPD were measured, then all monkeys were infected with 10^3 colony forming units of the Erdman strain of *Mycobacterium tuberculosis* by inserting the organisms directly in the right lungs of immunised animals. Twenty eight days following infection, body weight, temperature, ESR and LPA to PPD were measured in all monkeys, and the lungs were x-rayed to determine whether infection with live *M. tuberculosis* had resulted in the onset of pneumonia.

TABLE 1
COMPARISON OF INTRADERMAL AND INTRALUNG ROUTES OF IMMUNISATION

Group Number	**Identification Number of Monkey**	**Amount of Immunogen**	**Route of Immunisation**
1	S3101-E	0	-
(Controls)	3144-B	0	-
2	4080-B	500 µg	intradermal
(Immunised with heat-killed *M. vaccae*)	3586-B	500 µg	intradermal
3	3534-C	500 µg	intralung
(Immunised with heat-killed *M. vaccae*)	3160-A	500 µg	intralung
4			
(Immunised	3564-B	100 µg	intradermal
with culture filtrate)	3815-B	100 µg	intradermal
5			
(Immunised	4425-A	100 µg	intralung
with culture filtrate)	2779-D	100 µg	intralung

The results of these studies are provided below in Tables 2A-E and are summarized below:

Table 2A – Twenty-eight days after infection with *M. tuberculosis* Erdman, chest x-rays of control (non-immunised) monkeys revealed haziness over the right suprahilar regions of both animals, indicating the onset of pneumonia. This progressed and by day 56 post-infection x-rays indicated disease in both lungs. As expected, as disease progressed both control animals lost weight and showed significant LPA responses to PPD, indicating strong T cell reactivity to *M. tuberculosis*. The ESR measurements were variable but consistent with strong immune reactivity.

Table 2B – The two monkeys immunised twice with 500 μg *M. vaccae* intradermally showed no sign of lung disease 84 days post-infection with *M. tuberculosis*. The LPA responses to PPD indicated there was immune reactivity to *M. tuberculosis*, and both animals continued to gain weight, a consistent indication of a lack of disease.

Table 2C – The two monkeys immunised twice with 500 μg *M. vaccae* intralung showed almost identical results to those animals of Table 2B. There was no sign of lung disease 84 days post infection with *M. tuberculosis*, with consistent weight gains. Both animals showed LPA response to PPD in the immunisation phase (day 0-62) and post-infection, indicating strong T cell reactivity had developed as a result of using the lung as the route of immunisation and subsequent infection.

Immunisation twice with 500 μg of whole *M. vaccae* has consistently shown protective effects against subsequent infection with live *M. tuberculosis*. The data presented in Tables 2D and 2E show the effects of immunisation with 100 μg of *M. vaccae* culture filtrate. Monkeys immunised intradermally showed signs of developing disease 84 days post-infection, while in those immunised intralung, one animal showed disease after 56 days and one animal showed disease 84 days post-infection. This was a significant delay in disease onset indicating that the immunisation process had resulted in some protective immunity.

TABLE 2A

CONTROL MONKEYS

ID#	Days	Wt.Kgs	Temp.	ESR Mm/hr	LPA PPD10	LPA PPD1	X-Ray Remarks
S3101E	0	2.17	37.0	0	0.47	1.1	Negative
	34	1.88	37.3	ND	0.85	1.4	ND
	62	2.02	36.0	ND	1.3	1.5	ND
→ **Time of Infection**							
	28	2.09	38.0	2	1.3	3.7	Positive
	56	1.92	37.2	20	5.6	9.1	Positive
	84	1.81	37.5	8	4.7	5.6	Positive
	121	DIED					

ID#	Days	Wt.Kgs	Temp.	ESR Mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3144-B	0	2.05	36.7	0	0.87	1.8	Negative
	34	1.86	37.6	ND	2.2	1.4	ND
	62	1.87	36.5	ND	1.6	1.6	ND
→ **Time of Infection**							
	28	2.10	38.0	0	12	8.7	Positive
	56	1.96	37.6	0	29.6	21.1	Positive
	84	1.82	37.3	4	45.3	23.4	Positive
	131	DIED					

ND = Not Done

TABLE 2B

MONKEYS IMMUNISED WITH WHOLE HEAT-KILLED *M. VACCAE* (500 μg) INTRADERMAL

ID#	Days	Wt.Kgs	Temp.	ESR Mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
4080-B	0	2.05	37.1	1	1.1	0.77	Negative
	34	1.97	38.0	ND	1.7	1.4	ND
	62	2.09	36.7	ND	1.5	1.5	ND
→ **Time of Infection**							
	28	2.15	37.6	0	2.6	2.1	Negative
	56	2.17	37.6	0	8.2	7.6	Negative
	84	2.25	37.3	0	3.8	2.8	Negative
	178	DIED					

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3586-B	0	2.29	37.0	0	1.1	1.4	Negative
	34	2.22	38.0	ND	1.9	1.6	ND
	62	2.39	36.0	ND	1.3	1.6	ND
→ **Time of Infection**							
	28	2.31	38.2	0	3.2	2.6	Negative
	56	2.32	37.2	0	7.8	4.2	Negative
	84	2.81	37.4	0	3.4	1.8	Negative
	197	DIED					

ND = Not Done

TABLE 2C

MONKEYS IMMUNISED WITH WHOLE HEAT-KILLED *M. VACCAE* (500 μg) INTRALUNG

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3534-C	0	2.15	36.8	0	1.7	1.3	Negative
	34	2.00	37.8	ND	4.4	1.4	ND
	62	2.13	36.4	ND	3.2	1.9	ND
→ Time of Infection							
	28	2.38	37.7	0	1.9	2.6	Negative
	56	2.42	37.8	0	5.3	4.7	Negative
	84	2.46	37.1	1	3.1	3.2	Negative
	210	No sign of lung disease					Negative

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3160-A	0	2.17	37.3	0	1.2	0.79	Negative
	34	1.98	37.1	ND	3.9	7.8	ND
	62	2.17	36.9	ND	1.7	2.4	ND
→ Time of Infection							
	28	2.38	37.7	0	1.9	2.6	Negative
	56	2.42	37.8	0	5.3	4.7	Negative
	84	2.46	37.1	1	3.1	3.2	Negative
	210	Stable lung disease					Positive

ND = Not Done

TABLE 2D

MONKEYS IMMUNISED WITH CULTURE FILTRATE (100 μg) INTRADERMAL

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3564-B	0	2.40	37.2	0	1.4	1.4	Negative
	34	2.42	38.1	ND	3.3	2.7	ND
	62	2.31	37.1	ND	3.1	3.4	ND
→ Time of Infection							
	28	2.41	38.6	13	24	13.6	Negative
	56	2.38	38.6	0	12.7	12.0	Negative
	84	2.41	38.6	2	21.1	11.8	Positive
	140						Died

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
3815-B	0	2.31	36.3	0	1.0	1.4	Negative
	34	2.36	38.2	ND	1.9	2.0	ND
	62	2.36	36.4	ND	3.7	2.8	ND
→ Time of Infection							
	28	2.45	37.8	0	2.1	3.3	Negative
	56	2.28	37.3	4	8.0	5.6	Negative
	84	2.32	37.4	0	1.9	2.2	Positive
	210						Positive

ND = Not Done

TABLE 2E

MONKEYS IMMUNISED WITH CULTURE FILTRATE (100 μg) INTRALUNG

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
4425-A	0	2.05	36.0	0	0.35	1.2	Negative
	34	2.0	37.6	ND	3.0	2.4	ND
	62	2.11	37.6	ND	2.2	1.6	ND
→ Time of Infection							
	28	2.21	38.0	0	8.4	4.1	Negative
	56	2.11	37.6	0	23.9	17.7	Negative
	84	2.18	37.9	0	8.4	7.2	Positive
	210	Stable lung disease					Positive

ID#	Days	Wt.Kgs	Temp.	ESR mm/hr	LPA PPD 10μg	LPA PPD 1μg	X-Ray Remarks
2779-D	0	2,56	38.6	2	1.9	1.4	Negative
	28	2.55	37.9	ND	0.78	1.1	ND
	56	2.69	38.4	ND	1.3	1.5	ND
→ Time of Infection							
	56	2.25	39.0	24	ND	ND	Positive
	96						Died

ND = Not Done

EXAMPLE 3

EFFECT OF IMMUNISATION WITH *M. VACCAE* ON ASTHMA IN MICE

This example demonstrates that both heat-killed *M. vaccae* and DD-*M. vaccae*, when administered to mice via the intranasal route, are able to inhibit the development of an allergic immune response in the lungs. This was demonstrated in a mouse model of the asthma-like allergen specific lung disease. The severity of this allergic disease is reflected in the large numbers of eosinophils that accumulate in the lungs.

C57BL/6J mice were given 2 μg ovalbumin in 100 μl alum adjuvant by the intraperitoneal route at time 0 and 14 days, and subsequently given 100 μg ovalbumin in 50 μl phosphate buffered saline (PBS) by the intranasal route on day 28. The mice accumulated eosinophils in their lungs as detected by washing the airways of the anaesthetised mice with saline, collecting the washings (broncheolar lavage or BAL), and counting the numbers of eosinophils.

As shown in Figs. 2A and B, groups of seven mice administered either 10 or 1000 μg of heat-killed *M. vaccae* (Fig. 2A), or 10 , 100 or 200 μg of DD-*M. vaccae,* prepared as described below (Fig. 2B) intranasally 4 weeks before intranasal challenge with ovalbumin, had reduced percentages of eosinophils in the BAL cells collected 5 days after challenge with ovalbumin compared to control mice. Control mice were given intranasal PBS. Live *M. bovis* BCG at a dose of 2×10^5 colony forming units also reduced lung eosinophilia. The data in Figs. 2A and B show the mean and SEM per group of mice.

Figs. 2C and D show that mice given either 1000 μg of heat-killed *M. vaccae* (Fig. 2C) or 200 μg of DD-*M. vaccae* (Fig. 2D) intranasally as late as one week before challenge with ovalbumin had reduced percentages of eosinophils compared to control mice. In contrast, treatment with live BCG one week before challenge with ovalbumin did not inhibit the development of lung eosinophilia when compared with control mice.

As shown in Fig. 2E, immunisation with either 1 mg of heat-killed *M. vaccae* or 200 μg of DD-*M. vaccae,* given either intranasally (i.n.) or subcutaneously (s.c.), reduced lung

eosinophilia following challenge with ovalbumin when compared to control animals given PBS. In the same experiment, immunization with BCG of the Pasteur (BCG-P) and Connought (BCG-C) strains prior to challenge with ovalbumin also reduced the percentage of eosinophils in the BAL of mice.

Eosinophils are blood cells that are prominent in the airways in allergic asthma. The secreted products of eosinophils contribute to the swelling and inflammation of the mucosal linings of the airways in allergic asthma. The data shown in Figs. 2A-E indicate that treatment with heat-killed *M. vaccae* or DD-*M. vaccae* reduces the accumulation of lung eosinophils, and may be useful in reducing inflammation associated with eosinophilia in the airways, nasal mucosal and upper respiratory tract.

DD-*M.vaccae* depleted of mycolic acids and arabinogalactan

Mycolic acids were depleted from DD-*M.vaccae* by treatment with potassium hydroxide (0.5% KOH) in ethanol for 48 hours at 37°C. Mycolic acid depleted DD-*M.vaccae* cells were then washed with ethanol and ether and dried. Arabinogalactans were depleted from the KOH treated DD-*M.vaccae* by further treatment with 1% periodic acid in 3% acetic acid for 1 hr at room temperature followed by treatment with sodium borohydride 0.1M for 1 hour at room temperature. After arabinogalactan depletion, samples were washed with water and lyophilized. As shown in Table 3, both mycolate depleted DD-*M.vaccae* as well as mycolic acid and arabinogalactan depleted DD-*M.vaccae*, given intranasally to ovalbumin sensitized mice reduced the accumulation of eosinophils in the bronchoalveolar lavage fluid following challenge with ovalbumin.

Administration of heat-killed *M. vaccae,* DD-*M. vaccae* or DD-*M.vaccae* depleted of mycolic acids and arabinogalactan may therefore reduce the severity of asthma and diseases that involve similar immune abnormalities, such as allergic rhinitis.

In addition, serum samples were collected from mice in the experiment shown in Fig. 2E and antibodies to ovalbumin was measured by standard enzyme-linked immunoassay (EIA). As shown in Table 3A below, sera from mice infected with BCG had higher levels of ovalbumin specific IgG1 than sera from PBS controls. In contrast, mice

immunized with *M. vaccae* or DD-*M. vaccae* had similar or lower levels of ovalbumin-specific IgG1. As IgG1 antibodies are characteristic of a Th2 immune response, these results are consistent with the suppressive effects of heat-killed *M. vaccae* and DD-*M. vaccae* on the asthma-inducing Th2 immune responses.

TABLE 3

DECREASED LUNG EOSINOPHILIA IN MICE TREATED WITH MYCOLIC ACID DEPLETED DD-*M.VACCAE* OR MYCOLIC ACID AND ARABINOGALACTAN DEPLETED DD-*M.VACCAE*.

Treatment Group	% Eosinophils in BAL	
	Mean	S.E.M.
PBS	58.8	8.4
Mycolic acid depleted DD-*M.vaccae*	21.8	17.4
Mycolic acid and arabinogalactan depleted DD-*M.vaccae*	16.8	0.3

Note: At least 7 mice per group.

TABLE 3A

LOW ANTIGEN-SPECIFIC IgG1 SERUM LEVELS IN MICE IMMUNIZED WITH HEAT-KILLED *M. VACCAE* OR DD-*M. VACCAE*

Treatment Group	Serum IgG1	
	Mean	SEM
M.vaccae i.n.	185.00	8.3
M. vaccae s.c.	113.64	8.0
DD-*M. vaccae* i.n.	96.00	8.1
DD-*M. vaccae* s.c.	110.00	4.1
BCG, Pasteur	337.00	27.2
BCG, Connaught	248.00	46.1
PBS	177.14	11.4

Note: Ovalbumin-specific IgG1 was detected using anti-mouse IgG1 (Serotec). Group means are expressed as the reciprocal of the EU50 end point titre.

EXAMPLE 4

EFFECT OF IMMUNIZING MICE WITH *M.VACCAE*, DD-*M.VACCAE* OR RECOMBINANT *M. VACCAE* PROTEINS ON TUBERCULOSIS

This example illustrates the effect of immunization with heat-killed *M.vaccae*, DD-*M.vaccae* or recombinant *M. vaccae* proteins without additional adjuvants, or a combination of heat-killed *M.vaccae* with a pool of recombinant proteins derived from *M.vaccae*.

Mice were injected intraperitoneally with one of the following preparations on two occasions three weeks apart:

a) Phosphate buffered saline (PBS, control);
b) Heat-killed *M.vaccae* (500 ug);
c) DD-*M.vaccae* (50 ug);
d) A pool of recombinant proteins containing 15 ug of each of GV4P, GV7, GV9, GV27B, GV33 protein (prepared as described below); and
e) Heat-killed *M.vaccae* plus the pool of recombinant proteins

Three weeks after the last intraperitoneal immunization, the mice were infected with 5 X 10^5 live H37Rv *M.tuberculosis* organisms. After a further three weeks, the mice were sacrificed, and their spleens homogenized and assayed for colony forming units (CFU) of *M.tuberculosis* as an indicator of severity of infection.

Figs. 3A and 3B show data in which each point represents individual mice. The numbers of CFU recovered from control mice immunised with PBS alone were taken as the baseline. All data from experimental mice were expressed as number of logarithms of CFUs below the baseline for control mice (or log protection). As shown in Fig. 3A, mice immunized with heat-killed *M.vaccae* or DD-*M.vaccae* showed a mean reduction of >1 or 0.5 logs CFU, respectively.

As shown in Fig. 3B, the spleens of mice immunized with the pool of recombinant proteins containing GV4P, GV7, GV9, GV27B and GV33, had CFUs slightly less than control mice. However, when GV4P, GV7, GV9, GV27B and GV33 were given in combination with heat-killed *M.vaccae*, the reduction in CFUs exceeded a mean of >1.5 logs.

The data demonstrates the effectiveness of immunization with *M.vaccae*, DD-*M.vaccae* or recombinant proteins derived from *M.vaccae* against subsequent infection with tuberculosis, and further indicates that *M.vaccae*, DD-*M.vaccae* and recombinant proteins may be developed as vaccines against tuberculosis.

EXAMPLE 5

EFFECT OF INTRADERMAL INJECTION OF HEAT-KILLED *MYCOBACTERIUM VACCAE* ON PSORIASIS IN HUMAN PATIENTS

This example illustrates the effect of two intradermal injections of heat-killed *Mycobacterium vaccae* on psoriasis in human patients.

M. vaccae (ATCC Number 15483) was cultured in sterile Medium 90 (yeast extract, 2.5g/l; tryptone, 5g/l; glucose, 1 g/l) at 37 °C. The cells were harvested by centrifugation, and transferred into sterile Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) with glucose at 37 °C for one day. The medium was then centrifuged to pellet the bacteria, and the culture filtrate removed. The bacterial pellet was resuspended in phosphate buffered saline at a concentration of 10 mg/ml, equivalent to 10^{10} *M. vaccae* organisms per ml. The cell suspension was then autoclaved for 15 min at 120 °C and stored frozen at –20 °C. Prior to use the *M. vaccae* suspension was thawed, diluted to a concentration of 5 mg/ml in phosphate buffered saline, autoclaved for 15 min at 120 °C and 0.2 ml aliquoted under sterile conditions into vials for use in patients.

Twenty-four volunteer psoriatic patients, male and female, 15-61 years old with no other systemic diseases were admitted to treatment. Pregnant patients were not included. The patients had PASI scores of 12-35. The PASI score is a measure of the location, size and degree of skin scaling in psoriatic lesions on the body. A PASI score of above 12 reflects

widespread disease lesions on the body. The study commenced with a washout period of four weeks where the patients did not have systemic anti-psoriasis treatment or effective topical therapy.

The 24 patients were then injected intradermally with 0.1 ml *M. vaccae* (equivalent to 500 μg). This was followed three weeks later with a second intradermal injection with the same dose of *M. vaccae* (500 μg). Psoriasis was evaluated from four weeks before the first injection of heat-killed *M. vaccae* to twelve weeks after the first injection as follows:

A. The PASI scores were determined at -4, 0, 3, 6 and 12 weeks;

B. Patient questionnaires were completed at 0, 3, 6 and 12 weeks; and

C. Psoriatic lesions and each patient were photographed at 0, 3, 6, 9 and 12 weeks.

The data shown in Table 4 describe the age, sex and clinical background of each patient.

TABLE 4

Patient Data in the Study of the Effect of *M. vaccae* in Psoriasis

Code No.	Patient	Age/Sex	Duration of Disorder	Admission PASI Score
PS-001	D.C.	49/F	30 years	28.8
PS-002	E.S.	41/F	4 months	19.2
PS-003	M.G.	24/F	8 months	18.5
PS-004	D.B.	54/M	2 years	12.2
PS-005	C.E.	58/F	3 months	30.5
PS-006	M.G.	18/F	3 years	15.0
PS-007	L.M.	27/M	3 years	19.0
PS-008	C.C	21/F	1 month	12.2
PS-009	E.G	42/F	5 months	12.6
PS-010	J.G	28/M	7 years	19.4
PS-011	J.U	39/M	1 year	15.5
PS-012	C.S	47/M	3 years	30.9
PS-013	H.B	44/M	10 years	30.4
PS-014	N.J	41/M	17 years	26.7
PS-015	J.T	61/F	15 years	19.5
PS-016	L.P	44/M	5 years	30.2
PS-017	E.N	45/M	5 years	19.5
PS-018	E.L	28/F	19 years	16.0
PS-019	B.A	38/M	17 years	12.3
PS-020	P.P	58/F	1 year	13.6
PS-021	L.I	27/F	8 months	22.0
PS-022	A.C	20/F	7 months	26.5
PS-023	C.A	61/F	10 years	12.6
PS-024	F.T	39/M	15 years	29.5

All patients demonstrated a non-ulcerated, localised erythematous soft indurated reaction at the injection site. No side effects were noted, or complained of by the patients. The data shown in Table 5, below, are the measured skin reactions at the injection site, 48 hours, 72 hours and 7 days after the first and second injections of heat-killed *M. vaccae.* The data shown in Table 6, below, are the PASI scores of the patients at the time of the first injection of *M. vaccae* (Day 0) and 3, 6, 9, 12 and 24 weeks later.

It can clearly be seen that, by week 9 after the first injection of *M. vaccae*, 16 of 24 patients showed a significant improvement in PASI scores. Seven of fourteen patients who have completed 24 weeks of follow-up remained stable with no clinical sign of redevelopment of severe disease. These results demonstrate the effectiveness of multiple intradermal injections of inactivated *M. vaccae* in the treatment of psoriasis. PASI scores below 10 reflect widespread healing of lesions. Histopathology of skin biopsies indicated that normal skin structure is being restored. Only one of the first seven patients who have completed 28 weeks follow-up has had a relapse.

TABLE 5

Skin Reaction Measurements in Millimeter

Code No.	Time of Measurement					
	First Injection			Second Injection		
	48 hours	72 hours	7 days	48 hours	72 hours	7 days
PS-001	12x10	12x10	10x8	15x14	15x14	10x10
PS-002	18x14	20x18	18x14	16x12	18x12	15x10
PS-003	10x10	14x10	10x8	15x12	15x10	10x10
PS-004	14x12	22x18	20x15	20x20	20x18	14x10
PS-005	10x10	13x10	DNR	DNR	DNR	DNR
PS-006	10x8	10x10	6x4	12x10	15x15	10x6
PS-007	15x15	18x16	12x10	15x13	15x12	12x10
PS-008	18x18	13x12	12x10	18x17	15x10	15x10
PS-009	13x13	18x15	12x8	15x13	12x12	12x7
PS-010	13x11	15x15	8x8	12x12	12x12	5x5
PS-011	17x13	14x12	12x11	12x10	12x10	12x10
PS-012	17x12	15x12	9x9	10x10	10x6	8x6
PS-013	18x11	15x11	15x10	15x10	15x13	14x6
PS-014	15x12	15x11	15x10	13x12	14x10	8x5
PS-015	15x12	16x12	15x10	7x6	14x12	6x4
PS-016	6x5	6x6	6x5	8x8	9x8	9x6
PS-017	20x15	15x14	14x10	15x15	17x16	DNR
PS-018	14x10	10x8	10x8	12x12	10x10	10x10
PS-019	10x10	14x12	10x8	DNR	15x14	15x14
PS-020	15x12	15x15	12x15	15x15	14x12	13x12
PS-021	15x12	15x12	7x4	11x10	11x10	11x8
PS-022	12x10	10x8	10x8	15x12	13x10	10x8
PS-023	13x12	14x12	10x10	17x17	15x15	DNR

Code No.	Time of Measurement					
PS-024	10x10	10x10	10x8	10x8	8x7	8x7

DNR = Did not report.

TABLE 6

Clinical Status of Patients after Injection of *M. vaccae* (PASI Scores)

Code No.	Day 0	Week 3	Week 6	Week 9	Week 12	Week 24
PS-001	28.8	14.5	10.7	2.2	0.7	0
PS-002	19.2	14.6	13.6	10.9	6.2	0.6
PS-003	18.5	17.2	10.5	2.7	1.6	0
PS-004	12.2	13.4	12.7	7.0	1.8	0.2
PS-005*	30.5	DNR	18.7	DNR	DNR	0
PS-006	15.0	16.8	16.4	2.7	2.1	3.0
PS-007	19.0	15.7	11.6	5.6	2.2	0
PS-008	12.2	11.6	11.2	11.2	5.6	0
PS-009	12.6	13.4	13.9	14.4	15.3	13.0
PS-010	18.2	16.0	19.4	17.2	16.9	19.3
PS-011	17.2	16.9	16.7	16.5	16.5	15.5
PS-012	30.9	36.4	29.7	39.8**		
PS-013	19.5	19.2	18.9	17.8	14.7	17.8
PS-014	26.7	14.7	7.4	5.8	9.9	24.4***
PS-015	30.4	29.5	28.6	28.5	28.2	24.3
PS-016	30.2	16.8	5.7	3.2	0.8	3.3
PS-017	12.3	12.6	12.6	12.6	8.2	8.7
PS-018	16.0	13.6	13.4	13.4	13.2	12.8
PS-019	19.5	11.6	7.0	DNR	DNR	DNR
PS-020	13.6	13.5	12.4	12.7	12.4	4.4

PS-021	22.0	20.2	11.8	11.4	15.5	15.7
PS-022	26.5	25.8	20.7	11.1	8.3	5.6
PS-023	12.6	9.2	6.6	5.0	4.8	12.6
PS-024	29.5	27.5	20.9	19.0	29.8	21.2

- * Patient PS-005 received only one dose of autoclaved *M.vaccae*.
- ** Patient PS-012 removed from trial, drug (penicillin) induced dermatitis
- *** Patient PS-014 was revaccinated
- DNR = Did not report

Patients treated with *M.vaccae* may achieve remission (PASI score = 0). The remission or improvement of PASI score may be long lasting. By example, Patient PS-003 achieved remission by week 20 and was still in remission at week 80. Overall 13 of 24 patients showed a greater than 50% improvement in PASI scores.

Patient PS-001 achieved remission at week 16, relapsed at week 48 (PASI 2.7), was re-vaccinated with injections of *M.vaccae* and subsequently improved with PASI falling from 17.8 (Week 60) to 0.8 (week 84). Thus patients may benefit from repeated treatment.

EXAMPLE 6

EFFECT OF INTRADERMAL INJECTION OF DD-*M.VACCAE* ON PSORIASIS IN HUMAN PATIENTS

This example illustrates the effect of two intradermal injections of DD-*M. vaccae* on psoriasis.

Seven volunteer psoriatic patients, male and female, 18-45 years old with no other systemic diseases were admitted to treatment. Pregnant patients were not included. The patients had PASI scores of 12-24. As discussed above, the PASI score is a measure of the location, size and degree of skin scaling in psoriatic lesions on the body. A PASI score of

above 12 reflects widespread disease lesions on the body. The study commenced with a washout period of four weeks where the four patients did not have systemic antipsoriasis treatment or effective topical therapy. The seven patients were then injected intradermally with 0.1 ml DD-*M. vaccae* (equivalent to 100 μg). This was followed three weeks later with a second intradermal injection with the same dose of DD-*M. vaccae* (100 μg).

Psoriasis was evaluated from four weeks before the first injection of *M. vaccae* to six weeks after the first injection as follows:

A. the PASI scores were determined at -4, 0, 3 and 6 weeks;

B. patient questionnaires were completed at 0, 3 and 6 weeks; and

C. psoriatic lesions and each patient were photographed at 0 and 3 weeks.

The data shown in Table 7 describe the age, sex and clinical background of each patient.

TABLE 7

Patient Data in the Study of the Effect of DD-*M. vaccae* in Psoriasis

Code No.	Patient	Age/Sex	Duration of Disorder	Admission PASI Score
PS-025	A.S	25/F	2 years	12.2
PS-026	M.B	45/F	3 months	14.4
PS-027	A.G	34/M	14 years	24.8
PS-028	E.M	31/M	4 years	18.2
PS-029	A.L	44/M	5 months	18.6
PS-030	V.B	42/M	5years	21.3
PS-031	R.A	18/M	3 months	13.0

All patients demonstrated a non-ulcerated, localised erythematous soft indurated reaction at the injection site. No side effects were noted, or complained of by the patients. The data shown in Table 8 are the measured skin reactions at the injection site, 48 hours, 72 hours and 7 days after the first injection of DD-*M. vaccae,* and 48 hours and 72 hours after the second injection.

TABLE 8

Skin Reaction Measurements in Millimeters

Code No.	Time of Measurement				
	First Injection			Second Injection	
	48 hours	72 hours	7 days	48 hours	72 hours
PS-025	8x8	8x8	3x2	10x10	10x10
PS-026	12x12	12x12	8x8	DNR	14x14
PS-027	9x8	10x10	10x8	9x5	9x8
PS-028	10x10	10x10	10x8	10x10	10x10
PS-029	8x6	8x6	5x5	8x8	8x8
PS-030	14x12	14x14	10x10	12x10	12x10
PS-031	10x10	12x12	10x6	14x12	12x10

DNR = Did not report

The data shown in Table 9 are the PASI scores of the seven patients at the time of the first injection of DD-*M. vaccae* (Day 0), 3, 6, 12 and 24 weeks later.

TABLE 9

Clinical Status of Patients after Injection of DD-*M. vaccae* (PASI Scores)

Code No.	Day 0	Week 3	Week 6	Week 12	Week 24
PS-025	12·2	4·1	1.8	1.4	1.7
PS-026	14·4	11·8	6.0	6.9	1.4
PS-027	24·8	23·3	18.3	9.1	10.6
PS-028	18·2	24·1	28.6	Dropped	
PS-029	18.6	9.9	7.4	3.6	0.8
PS-030	21.3	15.7	13.9	16.5	13.6
PS-031	13.0	5.1	2.1	1.6	0.3

It can clearly be seen that by week 3 after the first injection of DD-*M. vaccae*, five patients showed a significant improvement in PASI scores. By week 24, six of seven patients showed a significant improvement in PASI score.

By way of example, Patient PS-031 went into remission (PASI score = 0) at week 32 and remained in remission when seen at week 48. The PASI score of patient PS-025 was reduced to less than 1 for more than 12 weeks. Upon an exacerbation of psoriasis (PASI = 15.8) at week 48, the patient was re-treated with DD-*M.vaccae* and improveded promptly with PASI scores falling to 6.8 and 0.6 at weeks 52 and 56 respectively.

Thus treatment of psoriasis with DD-*M.vaccae* may lead to disease remission or provide prolonged benefit. Patients may also benefit with repeated treatment.

EXAMPLE 7

PREPARATION OF COMPOSITIONS FROM *M.VACCAE*

This example illustrates the processing of different constituents of *M. vaccae*.

Preparation of Delipidated and Deglycolipidated (DD-) *M.vaccae* and Compositional Analysis

Heat-killed *M. vaccae* was prepared as described as above in Example 1. To prepare delipidated *M.vaccae*, the autoclaved *M.vaccae* was pelleted by centrifugation, the pellet washed with water, collected again by centrifugation and then freeze-dried. An aliquot of this freeze-dried *M.vaccae* was set aside and referred to as lyophilised *M.vaccae*. When used in experiments it was resuspended in PBS to the desired concentration. Freeze-dried *M. vaccae* was treated with chloroform/methanol (2:1) for 60 mins at room temperature to extract lipids, and the extraction was repeated once. The delipidated residue from chloroform/methanol extraction was further treated with 50% ethanol to remove glycolipids by refluxing for two hours. The 50% ethanol extraction was repeated two times. The pooled 50% ethanol extracts were used as a source of *M. vaccae* glycolipids (see below). The residue from the 50% ethanol extraction was freeze-dried and weighed. The amount of delipidated and deglycolipidated *M.vaccae* prepared was equivalent to 11.1% of the starting wet weight of

M.vaccae used. For bioassay, the delipidated and deglycolipidated *M. vaccae* (DD-*M. vaccae)*, was resuspended in phosphate-buffered saline by sonication, and sterilised by autoclaving.

The compositional analyses of heat-killed *M. vaccae* and DD-*M. vaccae* are presented in Table 9. Major changes are seen in the fatty acid composition and amino acid composition of DD-*M. vaccae* as compared to the insoluble fraction of heat-killed *M. vaccae*. The data presented in Table 9 show that the insoluble fraction of heat-killed *M.vaccae* contains 10% w/w of lipid, and the total amino acid content is 2750 nmoles/mg, or approximately 33% w/w. DD-*M. vaccae* contains 1.3% w/w of lipid and 4250 nmoles/mg amino acids, which is approximately 51% w/w.

TABLE 9

Compositional analyses of heat-killed *M. vaccae* and DD-*M. vaccae*

MONOSACCHARIDE COMPOSITION

sugar alditol	*M. vaccae*	DD-*M. vaccae*
Inositol	3.2%	1.7%
Ribitol *	1.7%	0.4%
Arabinitol	22.7%	27.0%
Mannitol	8.3%	3.3%
Galactitol	11.5%	12.6%
Glucitol	52.7%	55.2%

FATTY ACID COMPOSITION

Fatty acid	*M. vaccae*	DD-*M. vaccae*
C14:0	3.9%	10.0%
C16:0	21.1%	7.3%
C16:1	14.0%	3.3%
C18:0	4.0%	1.5%
C18:1*	1.2%	2.7%
C18:1w9	20.6%	3.1%
C18:1w7	12.5%	5.9%
C22:0	12.1%	43.0%
C24:1*	6.5%	22.9%

The insoluble fraction of heat-killed *M. vaccae* contains 10% w/w of lipid, and DD-*M. vaccae* contains 1.3% w/w of lipid.

AMINO ACID COMPOSITION

Nmoles/mg	*M. vaccae*	DD-*M. vaccae*
ASP	231	361
THR	170	266
SER	131	199
GLU	319	505
PRO	216	262
GLY	263	404
ALA	416	621
CYS*	24	26
VAL	172	272
MET*	72	94
ILE	104	171
LEU	209	340
TYR	39	75
PHE	76	132
GlcNH2	5	6
HIS	44	77
LYS	108	167
ARG	147	272

The total amino acid content of the insoluble fraction of heat-killed *M. vaccae* is 2750 nmoles/mg, or approximately 33% w/w. The total amino acid content of DD-*M. vaccae* is 4250 nmoles/mg, or approximately 51% w/w.

Comparison of composition of DD-*M. vaccae* with delipidated and deglycolipidated forms of *M. tuberculosis* and *M. smegmatis*

Delipidated and deglycolipidated *M. tuberculosis* and *M. smegmatis* were prepared using the procedure described above for delipidated and deglycolipidated *M. vaccae*. As indicated in Table 10, the profiles of the percentage composition of amino acids in *DD-M. vaccae,* DD-*M. tuberculosis* and DD-*M. smegmatis* showed no significant differences. However, the total amount of protein varied - the two batches of

DD-*M. vaccae* contained 34% and 55% protein, whereas DD-*M. tuberculosis* and DD-*M. smegmatis* contained 79% and 72% protein, respectively.

TABLE 10

Amino Acid Composition of Delipidated and Deglycolipidated Mycobacteria

Amino Acid	*DD-M.vaccae* Batch 1	DD-*M.vaccae* Batch 2	DD-*M.smegmatis*	DD-*M.tuberculosis*
Asp	9.5	9.5	9.3	9.1
Thr	6.0	5.9	5.0	5.3
Ser	5.3	5.3	4.2	3.3
Glu	11.1	11.2	11.1	12.5
Pro	6.1	5.9	7.5	5.2
Gly	9.9	9.7	9.4	9.8
Ala	14.6	14.7	14.6	14.2
Cys	0.5	0.5	0.3	0.5
Val	6.3	6.4	7.2	7.8
Met	1.9	1.9	1.9	1.9
Ile	3.6	3.5	4.1	4.7
Leu	7.8	7.9	8.2	8.3
Tyr	1.4	1.7	1.8	1.8
Phe	4.2	4.0	3.2	3.0
His	1.9	1.8	2.0	1.9
Lys	4.1	4.0	4.1	4.2
Arg	5.8	5.9	6.2	6.4
Total % Protein	**55.1**	**33.8**	**72.1**	**78.5**

Analysis of the monosaccharide composition shows significant differences between DD-*M. vaccae,* and DD-*M. tuberculosis* and DD-*M. smegmatis*. The monosaccharide composition of two batches of DD-*M. vaccae* was the same and differed from that of DD-*M. tuberculosis* and *M. smegmatis*. Specifically, DD-*M. vaccae* was found to contain free

glucose while both DD-*M. tuberculosis* and *M. smegmatis* contain glycerol, as shown in Table 11.

TABLE 11

Alditol Acetate	wt%	mol%
DD-M.vaccae Batch 1		
Inositol	0.0	0.0
Arabinose	54.7	59.1
Mannose	1.7	1.5
Glucose	31.1	28.1
Galactose	12.5	11.3
	100.0	100.0
DD-M.vaccae Batch 2		
Inositol	0.0	0.0
Arabinose	51.0	55.5
Mannose	2.0	1.8
Glucose	34.7	31.6
Galactose	12.2	11.1
	100.0	100.0
DD-M.smeg		
Inositol	0.0	0.0
Glycerol	15.2	15.5
Arabinose	69.3	70.7
Xylose	3.9	4.0
Mannose	2.2	1.9
Glucose	0.0	0.0
Galactose	9.4	8.0
	100.0	100.0
DD-Mtb		
Inositol	0.0	0.0
Glycerol	9.5	9.7
Arabinose	69.3	71.4
Mannose	3.5	3.0
Glucose	1.5	1.3
Galactose	12.4	10.7
	96.2	96.0

***M. vaccae* glycolipids**

The pooled 50% ethanol extracts described above were dried by rotary evaporation, redissolved in water, and freeze-dried. The amount of glycolipid recovered was 1.2% of the

starting wet weight of *M. vaccae* used. For bioassay, the glycolipids were dissolved in phosphate-buffered saline.

EXAMPLE 8

IMMUNE MODULATING PROPERTIES OF DELIPIDATED AND DEGLYCOLIPIDATED *M.VACCAE* AND RECOMBINANT PROTEINS FROM *M.VACCAE*

This example illustrates the immune modulating properties of different constituents of *M. vaccae*.

Production of Interleukin-12 from macrophages

Whole heat-killed *M. vaccae* and DD-*M. vaccae* were shown to have different cytokine stimulation properties. The stimulation of a Th1 immune response is enhanced by the production of interleukin-12 (IL-12) from macrophages. The ability of different *M. vaccae* preparations to stimulate IL-12 production was demonstrated as follows.

A group of C57BL/6J mice were injected intraperitoneally with DIFCO thioglycolate and after three days, peritoneal macrophages were collected and placed in cell culture with interferon-gamma for three hours. The culture medium was replaced and various concentrations of whole heat-killed (autoclaved) *M. vaccae*, lyophilized *M. vaccae*, DD-*M. vaccae* and *M. vaccae* glycolipids, prepared as described above, were added. After a further three days at 37 °C, the culture supernatants were assayed for the presence of IL-12 produced by macrophages. As shown in Fig. 4, the *M. vaccae* preparations stimulated the production of IL-12 from macrophages.

By contrast, these same *M. vaccae* preparations were examined for the ability to stimulate interferon-gamma production from Natural Killer (NK) cells. Spleen cells were prepared from Severe Combined Immunodeficient (SCID) mice. These populations contain 75-80% NK cells. The spleen cells were incubated at 37 °C in culture with different concentrations of heat-killed *M. vaccae*, DD-*M. vaccae*, or *M. vaccae* glycolipids. The data

shown in Fig. 5 demonstrates that, while heat-killed *M. vaccae* and *M. vaccae* glycolipids stimulate production of interferon-gamma, DD-*M. vaccae* stimulated relatively less interferon-gamma. The combined data from Figs. 4 and 5 indicate that, compared with whole heat-killed *M. vaccae*, DD-*M. vaccae* is a better stimulator of IL-12 than interferon gamma.

These findings demonstrate that removal of the lipid glycolipid constituents from *M. vaccae* results in the removal of molecular components that stimulate interferon-gamma from NK cells, thereby effectively eliminating an important cell source of a cytokine that has numerous harmful side-effects. DD-*M. vaccae* thus retains Th1 immune enhancing capacity by stimulating IL-12 production, but has lost the non-specific effects that may come through the stimulation of interferon-gamma production from NK cells.

The adjuvant effect of DD-*M. vaccae* and a number of *M. vaccae* recombinant antigens of the present invention, prepared as described below, was determined by measuring stimulation of IL-12 secretion from murine peritoneal macrophages. Figs. 6A, B, and C show data from separate experiments in which groups of C57BL/6 mice (Fig. 6A), BALB/c mice (Fig. 6B) or C3H/HeJ mice (Fig. 6C) were given DIFCO thioglycolate intraperitoneally. After three days, peritoneal macrophages were collected and placed in culture with interferon-gamma for three hours. The culture medium was replaced and various concentrations of *M. vaccae* recombinant proteins GVs-3 (GV-3), GV-4P (GV-4P), GVc-7 (GV-7), GV-23, GV-27, heat killed *M. vaccae*, DD-*M. vaccae* (referred to as delipidated *M. vaccae* in Figs. 6A, B and C), *M. vaccae* glycolipids or lipopolysaccharide were added. After three days at 37 °C, the culture supernatants were assayed for the presence of IL-12 produced by macrophages. As shown in Figs. 6A, B and C, the recombinant proteins and *M. vaccae* preparations stimulated the production of IL-12 from macrophages.

In a subsequent experiment, IFNγ-primed peritoneal macrophages from BALB/c mice were stimulated with 40 ug/ml of *M. vaccae* recombinant proteins in culture for 3 days and the presence of IL-12 produced by macrophages was assayed. As shown in Fig. 7, in these experiments IFNγ-primed macrophages produced IL-12 when cultured with a control protein, ovalbumin (ova). However, the recombinant proteins GV 24B, 38BP, 38AP, 27, 5, 27B, 3, 23

and 22B stimulated more than twice the amount of IL-12 detected in control macrophage cultures.

Detection of Nonspecific Immune Amplifier from Whole *M. vaccae* and the Culture Filtrate of *M. Vaccae*

M. vaccae culture supernatant (S/N), killed *M. vaccae,* delipidated *M. vaccae* and delipidated and deglycolipidated *M. vaccae* (DD-*M. vaccae*), prepared as described above, were tested for adjuvant activity in the generation of a cytotoxic T cell immune response to ovalbumin, a structurally unrelated protein, in the mouse. This anti-ovalbumin-specific cytotoxic response was detected as follows. C57BL/6 mice (2 per group) were immunized by the intraperitoneal injection of 100 μg of ovalbumin with the following test adjuvants: autoclaved *M. vaccae*; delipidated *M. vaccae*; delipidated *M. vaccae* with glycolipids also extracted (DD-*M. vaccae*) and proteins extracted with SDS; the SDS protein extract treated with Pronase (an enzyme which degrades protein); whole *M. vaccae* culture filtrate; and heat-killed *M. tuberculosis* or heat-killed *M. bovis* BCG, *M. phlei* or *M. smegmatis* or *M. vaccae* culture filtrate. After 10 days, spleen cells were stimulated *in vitro* for a further 6 days with E.G7 cells which are EL4 cells (a C57BL/6-derived T cell lymphoma) transfected with the ovalbumin gene and thus express ovalbumin. The spleen cells were then assayed for their ability to kill non-specifically EL4 target cells or to kill specifically the E.G7 ovalbumin expressing cells. Killing activity was detected by the release of ^{51} Chromium with which the EL4 and E.G7 cells have been labelled (100 μCi per $2x10^6$), prior to the killing assay. Killing or cytolytic activity is expressed as % specific lysis using the formula:

$$\frac{\text{cpm in test cultures} - \text{cpm in control cultures}}{\text{total cpm} - \text{cpm in control cultures}} \times 100\%$$

It is generally known that ovalbumin-specific cytotoxic cells are generated only in mice immunized with ovalbumin with an adjuvant but not in mice immunized with ovalbumin alone.

The diagrams that make up Fig. 7 show the effect of various *M. vaccae* derived adjuvant preparations on the generation of cytotoxic T cells to ovalbumin in C57BL/6 mice. As shown in Fig. 7A, cytotoxic cells were generated in mice immunized with (i) 10 μg, (ii) 100 μg or (iii) 1 mg of autoclaved *M. vaccae* or (iv) 75 μg of *M. vaccae* culture filtrate. Fig. 7B shows that cytotoxic cells were generated in mice immunized with (i) 1 mg whole autoclaved *M. vaccae* or (ii) 1 mg delipidated and deglycolipidated (DD-) *M. vaccae*. As shown in Fig. 7C(i), cytotoxic cells were generated in mice immunized with 1 mg whole autoclaved *M. vaccae*; Fig. 7C(ii) shows the active material in *M. vaccae* soluble proteins extracted with SDS from DD-*M. vaccae*. Fig. 7C(iii) shows that active material in the adjuvant preparation of Fig. 7C(ii) was destroyed by treatment with the proteolytic enzyme Pronase. By way of comparison, 100 μg of the SDS-extracted proteins had significantly stronger immune-enhancing ability (Fig. 7C(ii)) than did 1 mg whole autoclaved *M. vaccae* (Fig. 7C(i)).

Mice immunized with 1 mg heat-killed *M. vaccae* (Fig. 7D(i)) generated cytotoxic cells to ovalbumin, but mice immunized separately with 1 mg heat-killed *M. tuberculosis* (Fig. 7D(ii)), 1 mg *M. bovis* BCG (Fig. 7D(iii)), 1 mg *M. phlei* (Fig. 7D(iv)), or 1 mg *M. smegmatis* (Fig. 7D(v)) failed to generate cytotoxic cells.

These findings demonstrate that heat-killed *M. vaccae* and DD-*M. vaccae* have adjuvant properties not seen in other mycobacteria. Furthermore, delipidation and deglycolipidation of *M. vaccae* removes an NK cell-stimulating activity but does not result in a loss of T-cell stimulating activity.

In a separate experiment, mice immunised with ovalbumin plus 200 ug of DD-*M.vaccae* depleted of mycolic acids and arabinogalactan, were also able to generate cytotoxic cells (28% to 46% maximum specific lysis compared with <8% specific lysis for control mice immunised with ovalbumin alone).

The *M. vaccae* culture filtrate described above was fractionated by iso-electric focusing and the fractions assayed for adjuvant activity in the anti-ovalbumin-specific cytotoxic response assay in C57BL/6 mice as described above. Peak adjuvant activities were

demonstrated in fractions corresponding to pI of 4.2-4.32 (fraction nos. 7-9), 4.49-4.57 (fraction nos. 13-17) and 4.81-5.98 (fraction nos. 23-27).

Identifcation of proteins in DD-*M. vaccae* by antibodies

BALB/c mice were immunised intra-peritoneally with 50 ug of DD-*M. vaccae* once a week for 5 weeks. At the 6th week mice were sacrificed and their serum collected. The sera were tested for antibodies to recombinant *M. vaccae*-derived proteins, prepared as described below, in standard enzyme-linked immunoassays.

The antisera did not react with several *M. vaccae* recombinant proteins nor with ovalbumin, which served as an irrelevant negative control protein in the enzyme-linked assays (data not shown). Antisera from mice immunised with DD-*M. vaccae* reacted with *12 M. vaccae*-derived GV antigens. The results are shown in Table 12 below. The antisera thus identified GV3, 5P, 5, 7, 9, 22B, 24, 27, 27A, 27B, 33 and 45 as being present in DD-*M. vaccae*.

TABLE 12
Reactivity of DD-*M. vaccae* antiserum with *M.vaccae*-derived GV antigens

GV Antigen	3	5P	5	7	9	22B	24	27	27A	27B	33	45
Reactivity*	10^3	10^3	10^3	10^2	10^4	10^3	10^4	10^6	10^5	10^6	10^4	10^4

*Expressed as highest dilution of serum from DD-*M.vaccae* immunised mice showing greater activity than serum from non-immunised mice.

Proteins in DD-*M.vaccae* identified by T cell responses

BALB/c mice were injected in each footpad with 100 ug DD-*M.vaccae* in combination with incomplete Freund's adjuvant and 10 days later were sacrificed to obtain popliteal lymph node cells. The cells from immunized and non-immunized control mice were stimulated *in vitro* with recombinant *M. vaccae*-derived GV proteins. After 3 days, cell proliferation and IFNγ production were assessed.

T cell proliferative responses of lymph node cells from DD-*M.vaccae* immunized mice to GV proteins.

Lymph node cells from DD-*M. vaccae*-immunized mice did not proliferate in response to an irrelevant protein, ovalbumin, (data not shown). As shown in Table 13, lymph node cells from immunized mice showed proliferative responses to GV 3, 7, 9, 23, 27, 27B, and 33. The corresponding cells from non-immunized mice did not proliferate in response to these GV proteins suggesting that mice immunized with DD-*M. vaccae* have been immunized with these proteins. Thus, the *M.vaccae* derived proteins GV 3, 7, 9, 23, 27, 27B and 33 are likely to be present in DD-*M.vaccae*.

TABLE 13

Proliferative responses of lymph node cells from DD-*M.vaccae*-immunised mice and control mice to GV proteins *in vitro*

GV protein	**Stimulation index* observed in the presence of GV proteins at 50 μg/ml**	
	DD-*M.vaccae* immunised mice	**Control mice**
GV3	4.63	1.52
GV7	3.32	1.27
GV9	6.48	2.64
GV23	4.00	1.76
GV27	5.13	1.40
GV27B	7.52	1.48
GV33	3.31	1.45

*Stimulation index = cpm from tritiated Thymidine uptake in presence of GV protein/cpm in absence of GV protein

IFNγ production by lymph node cells from DD-*M. vaccae* immunized mice following *in vitro* challenge with GV proteins

Lymph node cells from non-immunized mice did not produce IFNγ upon stimulation with GV proteins. As shown in Table 14 below, lymph node cells from DD-*M.vaccae* immunized mice secrete IFNγ in a dose dependent manner when stimulated with GV 3, 5, 23, 27A, 27B, 33, 45 or 46, suggesting that the mice have been immunized with these proteins. No IFNγ production was detectable when cells from immunized mice were stimulated with the irrelevant protein, ovalbumin (data not shown). The proteins GV 3, 5, 23, 27A, 27B, 33, 45 and 46 are thus likely to be present in DD-*M. vaccae*.

TABLE 14

Production of IFNγ by popliteal lymph node cells from DD-*M.vaccae*-immunised mice following *in vitro* challenge with GV protein

GV protein or control	**IFNγ (ng/ml)**		
	Dose of GV protein used *in vitro* (μg/ml)		
	50	10	2
GV-3	8.22 ± 3.73	ND	ND
GV-4P	ND	ND	ND
GV-5	8.90 ± 4.54	0.57 ± 0.40	ND
GV-5P	ND	ND	ND
GV-7	ND	ND	ND
GV-9	ND	ND	ND
GV-13	1.64 ± 0.40	ND	ND
GV-14	ND	ND	ND
GV-14B	ND	ND	ND
GV-22B	20.15 ± 1.96	4.34 ± 0.02	ND
GV-23	41.38 ± 6.69	6.97 ± 2.78	ND
GV-24B	ND	ND	ND
GV-27	46.86 ± 17.14	33.06 ± 17.61	10.14 ± 3.01
GV-27A	7.25 ± 4.36	ND	ND
GV-27B	100.36 ± 37.84	33.03 ± 7.54	14.33 ± 1.01
GV-29	5.93 ± 0.47	ND	ND
GV-33	9.82 ± 4.64	ND	ND
GV-38AP	1.44 ± 1.20	ND	ND
GV-38BP	5.62 ± 0.70	ND	ND
GV-42	ND	ND	ND
GV-44	ND	ND	ND

DD-M.vaccae	109.59 ± 15.48	90.23 ± 6.48	65.16 ± 3.68
M. vaccae	68.89 ± 4.38	67.91 ± 7.92	48.92 ± 3.86

ND = Not Detectable

Proteins in DD-*M.vaccae* as non-specific immune amplifiers

In subsequent experiments, the five proteins GV27, 27A, 27B, 23 and 45 were used as non-specific immune amplifiers with ovalbumin antigen to immunize mice as described above in Example 6. As shown in Figure 12, 50 ug of any one of the recombinant proteins GV27, 27A, 27B, 23 and 45, when injected with 50-100 ug of ovalbumin, demonstrated adjuvant properties in being able to generate cytotoxic cells to ovalbumin.

EXAMPLE 9

AUTOCLAVED *M. VACCAE* GENERATES CYTOTOXIC CD8 T CELLS AGAINST *M. TUBERCULOSIS* INFECTED MACROPHAGES

This example illustrates the ability of killed *M. vaccae* to stimulate cytotoxic CD8 T cells which preferentially kill macrophages that have been infected with *M. tuberculosis*.

Mice were immunized by the intraperitoneal injection of 500 μg of killed *M. vaccae* which was prepared as described in Example 1. Two weeks after immunization, the spleen cells of immunized mice were passed through a CD8 T cell enrichment column (R&D Systems, St. Paul, MN, USA). The spleen cells recovered from the column have been shown to be enriched up to 90% CD8 T cells. These T cells, as well as CD8 T cells from spleens of non-immunized mice, were tested for their ability to kill uninfected macrophages or macrophages which have been infected with *M. tuberculosis*.

Macrophages were obtained from the peritoneal cavity of mice five days after they have been given 1 ml of 3% thioglycolate intraperitoneally. The macrophages were infected overnight with *M. tuberculosis* at the ratio of 2 mycobacteria per macrophage. All macrophage preparations were labelled with ^{51}Chromium at 2 μCi per 10^4 macrophages. The macrophages were then cultured with CD8 T cells overnight (16 hours) at killer to target

ratios of 30:1. Specific killing was detected by the release of ^{51}Chromium and expressed as % specific lysis, calculated as in Example 5.

The production of IFN-γ and its release into medium after 3 days of co-culture of CD8 T cells with macrophages was measured using an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with a rat monoclonal antibody directed to mouse IFN-γ (Pharmigen, San Diego, CA, USA) in PBS for 4 hours at 4 °C. Wells were blocked with PBS containing 0.2% Tween 20 for 1 hour at room temperature. The plates were then washed four times in PBS containing 0.2% Tween 20, and samples diluted 1:2 in culture medium in the ELISA plates were incubated overnight at room temperature. The plates were again washed, and a biotinylated monoclonal rat anti-mouse IFN-γ antibody (Pharmigen), diluted to 1 μg/ml in PBS, was added to each well. The plates were then incubated for 1 hour at room temperature, washed, and horseradish peroxidase-coupled avidin D (Sigma A-3151) was added at a 1:4,000 dilution in PBS. After a further 1 hour incubation at room temperature, the plates were washed and OPD substrate added. The reaction was stopped after 10 min with 10% (v/v) HCl. The optical density was determined at 490 nm. Fractions that resulted in both replicates giving an OD two-fold greater than the mean OD from cells cultured in medium alone were considered positive.

As shown in Table 15, CD8 T cells from spleens of mice immunized with *M. vaccae* were cytotoxic for macrophages infected with *M. tuberculosis* and did not lyse uninfected macrophages. The CD8 T cells from non-immunized mice did not lyse macrophages. CD8 T cells from naive or non-immunized mice do produce IFN-γ when cocultured with infected macrophages. The amount of IFN-γ produced in coculture was greater with CD8 T cells derived from *M. vaccae* immunized mice.

TABLE 15

EFFECT WITH *M. TUBERCULOSIS* INFECTED AND UNINFECTED MACROPHAGES

	% Specific Lysis of Macrophages		**IFN-γ (ng/ml)**	
CD8 T cells	uninfected	infected	uninfected	infected
Control	0	0	0.7	24.6
M. vaccae Immunized	0	95	2.2	43.8

EXAMPLE 10

PURIFICATION AND CHARACTERIZATION OF POLYPEPTIDES FROM *M. VACCAE* CULTURE FILTRATE

This example illustrates the preparation of *M. vaccae* soluble proteins from culture filtrate. Unless otherwise noted, all percentages in the following example are weight per volume.

M. vaccae (ATCC Number 15483) was cultured in sterile Medium 90 at 37 °C. The cells were harvested by centrifugation, and transferred into sterile Middlebrook 7H9 medium with glucose at 37 °C for one day. The medium was then centrifuged (leaving the bulk of the cells) and filtered through a 0.45 μm filter into sterile bottles.

The culture filtrate was concentrated by lyophilization, and redissolved in MilliQ water. A small amount of insoluble material was removed by filtration through a 0.45μm membrane. The culture filtrate was desalted by membrane filtration in a 400 ml Amicon stirred cell which contained a 3kDa molecular weight cut-off (MWCO) membrane. The pressure was maintained at 50 psi using nitrogen gas. The culture filtrate was repeatedly concentrated by membrane filtration and diluted with water until the conductivity of the

sample was less than 1.0 mS. This procedure reduced the 20 l volume to approximately 50 ml. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

The desalted culture filtrate was fractionated by ion exchange chromatography on a column of Q-Sepharose (Pharmacia Biotech, Uppsala, Sweden) (16 X 100 mm) equilibrated with 10mM Tris HCl buffer pH 8.0. Polypeptides were eluted with a linear gradient of NaCl from 0 to 1.0 M in the above buffer system. The column eluent was monitored at a wavelength of 280 nm.

The pool of polypeptides eluting from the ion exchange column was concentrated in a 400 ml Amicon stirred cell which contained a 3 kDa MWCO membrane. The pressure was maintained at 50 psi using nitrogen gas. The polypeptides were repeatedly concentrated by membrane filtration and diluted with 1% glycine until the conductivity of the sample was less than 0.1 mS.

The purified polypeptides were then fractionated by preparative isoelectric focusing in a Rotofor device (Bio-Rad, Hercules, CA, USA). The pH gradient was established with a mixture of Ampholytes (Pharmacia Biotech) comprising 1.6% pH 3.5-5.0 Ampholytes and 0.4% pH 5.0 - 7.0 Ampholytes. Acetic acid (0.5 M) was used as the anolyte, and 0.5 M ethanolamine as the catholyte. Isoelectric focusing was carried out at 12W constant power for 6 hours, following the manufacturer's instructions. Twenty fractions were obtained.

Fractions from isoelectric focusing were combined, and the polypeptides were purified on a Vydac C4 column (Separations Group, Hesperia, CA, USA) 300 Angstrom pore size, 5 micron particle size (10 x 250 mm). The polypeptides were eluted from the column with a linear gradient of acetonitrile (0-80% v/v) in 0.05% (v/v) trifluoroacetic acid (TFA). The flow-rate was 2.0 ml/min and the HPLC eluent was monitored at 220 nm. Fractions containing polypeptides were collected to maximize the purity of the individual samples.

Relatively abundant polypeptide fractions were rechromatographed on a Vydac C4 column (Separations Group) 300 Angstrom pore size, 5 micron particle size (4.6 x 250 mm). The polypeptides were eluted from the column with a linear gradient from 20-60% (v/v) of acetonitrile in 0.05% (v/v) TFA at a flow-rate of 1.0 ml/min. The column eluent was

monitored at 220 nm. Fractions containing the eluted polypeptides were collected to maximise the purity of the individual samples. Approximately 20 polypeptide samples were obtained and they were analysed for purity on a polyacrylamide gel according to the procedure of Laemmli (Laemmli, U. K., Nature 277:680-685, 1970).

The polypeptide fractions which were shown to contain significant contamination were further purified using a Mono Q column (Pharmacia Biotech) 10 micron particle size (5 x 50 mm) or a Vydac Diphenyl column (Separations Group) 300 Angstrom pore size, 5 micron particle size (4.6 x 250 mm). From a Mono Q column, polypeptides were eluted with a linear gradient from 0-0.5 M NaCl in 10 mM Tris HCl pH 8.0. From a Vydac Diphenyl column, polypeptides were eluted with a linear gradient of acetonitrile (20-60% v/v) in 0.1% TFA. The flow-rate was 1.0 ml/min and the column eluent was monitored at 220 nm for both columns. The polypeptide peak fractions were collected and analysed for purity on a 15% polyacrylamide gel as described above.

For sequencing, the polypeptides were individually dried onto Biobrene™ (Perkin Elmer/Applied BioSystems Division, Foster City, CA)-treated glass fiber filters. The filters with polypeptide were loaded onto a Perkin Elmer/Applied BioSystems Procise 492 protein sequencer and the polypeptides were sequenced from the amino terminal end using traditional Edman chemistry. The amino acid sequence was determined for each polypeptide by comparing the retention time of the PTH amino acid derivative to the appropriate PTH derivative standards.

Internal sequences were also determined on some antigens by digesting the antigen with the endoprotease Lys-C, or by chemically cleaving the antigen with cyanogen bromide. Peptides resulting from either of these procedures were separated by reversed-phase HPLC on a Vydac C18 column using a mobile phase of 0.05% (v/v) trifluoroacetic acid with a gradient of acetonitrile containing 0.05% (v/v) TFA (1%/min). The eluent was monitored at 214 nm. Major internal peptides were identified by their UV absorbance, and their N-terminal sequences were determined as described above.

Using the procedures described above, six soluble *M. vaccae* antigens, designated GVc-1, GVc-2, GVc-7, GVc-13, GVc-20 and GVc-22, were isolated. Determined N-terminal

and internal sequences for GVc-1 are shown in SEQ ID NOS: 1, 2 and 3, respectively; the N-terminal sequence for GVc-2 is shown in SEQ ID NO: 4; internal sequences for GVc-7 are shown in SEQ ID NOS: 5-8; internal sequences for GVc-13 are shown in SEQ ID NOS: 9-11; internal sequence for GVc-20 is shown in SEQ ID NO: 12; and N-terminal and internal sequences for GVc-22 are shown in SEQ ID NO: 56-59, respectively. Each of the internal peptide sequences provided herein begins with an amino acid residue which is assumed to exist in this position in the polypeptide, based on the known cleavage specificity of cyanogen bromide (Met) or Lys-C (Lys).

Three additional polypeptides, designated GVc-16, GVc-18 and GVc-21, were isolated employing a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) purification step in addition to the preparative isoelectric focusing procedure described above. Specifically, fractions comprising mixtures of polypeptides from the preparative isoelectric focusing purification step previously described were purified by preparative SDS-PAGE on a 15% polyacrylamide gel. The samples were dissolved in reducing sample buffer and applied to the gel. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer pH 11 containing 10% (v/v) methanol. The transferred protein bands were identified by staining the PVDF membrane with Coomassie blue. Regions of the PVDF membrane containing the most abundant polypeptide species were cut out and directly introduced into the sample cartridge of the Perkin Elmer/Applied BioSystems Procise 492 protein sequencer. Protein sequences were determined as described above. The N-terminal sequences for GVc-16, GVc-18 and GVc-21 are provided in SEQ ID NOS: 13, 14 and 15, respectively.

Additional antigens, designated GVc-12, GVc-14, GVc-15, GVc-17 and GVc-19, were isolated employing a preparative SDS-PAGE purification step in addition to the chromatographic procedures described above. Specifically, fractions comprising a mixture of antigens from the Vydac C4 HPLC purification step previously described were fractionated by preparative SDS-PAGE on a polyacrylamide gel. The samples were dissolved in non-reducing sample buffer and applied to the gel. The separated proteins were transferred to a

PVDF membrane by electroblotting in 10 mM CAPS buffer, pH 11 containing 10% (v/v) methanol. The transferred protein bands were identified by staining the PVDF membrane with Coomassie blue. Regions of the PVDF membrane containing the most abundant polypeptide species were cut out and directly introduced into the sample cartridge of the Perkin Elmer/Applied BioSystems Procise 492 protein sequencer. Protein sequences were determined as described above. The determined N-terminal sequences for GVc-12, GVc-14, GVc-15, GVc-17 and GVc-19 are provided in SEQ ID NOS: 16-20, respectively.

All of the above amino acid sequences were compared to known amino acid sequences in the SwissProt data base (version R32) using the GeneAssist system. No significant homologies to the amino acid sequences GVc-2 to GVc-22 were obtained. The amino acid sequence for GVc-1 was found to bear some similarity to sequences previously identified from *M. bovis* and *M. tuberculosis*. In particular, GVc-1 was found to have some homology with *M. tuberculosis* MPT83, a cell surface protein, as well as MPT70. These proteins form part of a protein family (Harboe et al., *Scand. J. Immunol. 42*:46-51, 1995).

Subsequent studies led to the isolation of DNA sequences for GVc-13, GVc-14 and GVc-22 (SEQ ID NO: 142, 107 and 108, respectively). The corresponding predicted amino acid sequences for GVc-13, GVc-14 and GVc-22 are provided in SEQ ID NO: 143, 109 and 110, respectively. The determined DNA sequence for the full length gene encoding GVc-13 is provided in SEQ ID NO: 195, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 196.

Further studies with GVc-22 suggested that only a part of the gene encoding GVc-22 was cloned. When sub-cloned into the expression vector pET16, no protein expression was obtained. Subsequent screening of the *M. vaccae Bam*HI genomic DNA library with the incomplete gene fragment led to the isolation of the complete gene encoding GVc-22. To distinguish between the full-length clone and the partial GVc-22, the antigen expressed by the full-length gene was called GV-22B. The determined nucleotide sequence of the gene encoding GV-22B and the predicted amino acid sequence are provided in SEQ ID NOS: 144 and 145 respectively.

Amplifications primers AD86 and AD112 (SEQ ID NO: 60 and 61, respectively) were designed from the amino acid sequence of GVc-1 (SEQ ID NO: 1) and the *M. tuberculosis* MPT70 gene sequence. Using these primers, a 310 bp fragment was amplified from *M. vaccae* genomic DNA and cloned into *Eco*RV-digested vector pBluescript II SK^+ (Stratagene). The sequence of the cloned insert is provided in SEQ ID NO: 62. The insert of this clone was used to screen a *M. vaccae* genomic DNA library constructed in lambda ZAP-Express (Stratagene, La Jolla, CA). The clone isolated contained an open reading frame with homology to the *M. tuberculosis* antigen MPT83 and was re-named GV-1/83. This gene also had homology to the *M. bovis* antigen MPB83. The determined nucleotide sequence and predicted amino acid sequences are provided in SEQ ID NOS: 146 and 147 respectively.

From the amino acid sequences provided in SEQ ID NOS: 1 and 2, degenerate oligonucleotides EV59 and EV61 (SEQ ID NOS: 148 and 149 respectively) were designed. Using PCR, a 100 bp fragment was amplified, cloned into plasmid pBluescript II SK^+ and sequenced (SEQ ID NO: 150) following standard procedures (Sambrook et al. *Ibid*). The cloned insert was used to screen a *M. vaccae* genomic DNA library constructed in lambda ZAP-Express. The clone isolated had homology to *M. tuberculosis* antigen MPT70 and *M. bovis* antigen MPB70, and was named GV-1/70. The determined nucleotide sequence and predicted amino acid sequence for GV-1/70 are provided in SEQ ID NOS: 151 and 152 respectively.

For expression and purification, the genes encoding GV1/83, GV1/70, GVc-13, GVc-14 and GV-22B were sub-cloned into the expression vector pET16 (Novagen, Madison, WI). Expression and purification were performed according to the manufacturer's protocol.

The purified polypeptides were screened for the ability to induce T-cell proliferation and IFN-γ in peripheral blood cells from immune human donors. These donors were known to be PPD (purified protein derivative from *M. tuberculosis*) skin test positive and their T cells were shown to proliferate in response to PPD. Donor PBMCs and crude soluble proteins from *M. vaccae* culture filtrate were cultured in medium comprising RPMI 1640 supplemented with 10% (v/v) autologous serum, penicillin (60 μg/ml), streptomycin (100 μg/ml), and glutamine (2 mM).

After 3 days, 50 μl of medium was removed from each well for the determination of IFN-γ levels, as described below. The plates were cultured for a further 4 days and then pulsed with 1μCi/well of tritiated thymidine for a further 18 hours, harvested and tritium uptake determined using a scintillation counter. Fractions that stimulated proliferation in both replicates two-fold greater than the proliferation observed in cells cultured in medium alone were considered positive.

IFN-γ was measured using an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with a mouse monoclonal antibody directed to human IFN-γ (Endogen, Wobural, MA) 1 μg/ml phosphate-buffered saline (PBS) for 4 hours at 4 °C. Wells were blocked with PBS containing 0.2% Tween 20 for 1 hour at room temperature. The plates were then washed four times in PBS/0.2% Tween 20, and samples diluted 1:2 in culture medium in the ELISA plates were incubated overnight at room temperature. The plates were again washed, and a biotinylated polyclonal rabbit anti-human IFN-γ serum (Endogen), diluted to 1 μg/ml in PBS, was added to each well. The plates were then incubated for 1 hour at room temperature, washed, and horseradish peroxidase-coupled avidin A (Vector Laboratories, Burlingame, CA) was added at a 1:4,000 dilution in PBS. After a further 1 hour incubation at room temperature, the plates were washed and orthophenylenediamine (OPD) substrate added. The reaction was stopped after 10 min with 10% (v/v) HCl. The optical density (OD) was determined at 490 nm. Fractions that resulted in both replicates giving an OD two-fold greater than the mean OD from cells cultured in medium alone were considered positive.

Examples of polypeptides containing sequences that stimulate peripheral blood mononuclear cells (PBMC) T cells to proliferate and produce IFN-γ are shown in Table 16, wherein (-) indicates a lack of activity, (+/-) indicates polypeptides having a result less than twice higher than background activity of control media, (+) indicates polypeptides having activity two to four times above background, and (++) indicates polypeptides having activity greater than four times above background.

TABLE 16

Antigen	Proliferation	IFN-γ
GVc-1	++	+/-
GVc-2	+	++
GVc-7	+/-	-
GVc-13	+	++
GVc-14	++	+
GVc-15	+	+
GVc-20	+	+

EXAMPLE 11

PURIFICATION AND CHARACTERISATION OF POLYPEPTIDES FROM *M. VACCAE* CULTURE FILTRATE BY 2-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

M. vaccae soluble proteins were isolated from culture filtrate using 2-dimensional polyacrylamide gel electrophoresis as described below. Unless otherwise noted, all percentages in the following example are weight per volume.

M. vaccae (ATCC Number 15483) was cultured in sterile Medium 90 at 37 °C. *M. tuberculosis* strain H37Rv (ATCC number 27294) was cultured in sterile Middlebrook 7H9 medium with Tween 80 and oleic acid/albumin/dextrose/catalase additive (Difco Laboratories, Detroit, Michigan). The cells were harvested by centrifugation, and transferred into sterile Middlebrook 7H9 medium with glucose at 37 °C for one day. The medium was then centrifuged (leaving the bulk of the cells) and filtered through a 0.45 μm filter into sterile bottles. The culture filtrate was concentrated by lyophilisation, and redissolved in MilliQ water. A small amount of insoluble material was removed by filtration through a 0.45 μm membrane filter.

The culture filtrate was desalted by membrane filtration in a 400 ml Amicon stirred cell which contained a 3 kDa MWCO membrane. The pressure was maintained at 60 psi using nitrogen gas. The culture filtrate was repeatedly concentrated by membrane filtration and diluted with water until the conductivity of the sample was less than 1.0 mS. This procedure reduced the 20 l volume to approximately 50 ml. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

The desalted culture filtrate was fractionated by ion exchange chromatography on a column of Q-Sepharose (Pharmacia Biotech) (16 x 100 mm) equilibrated with 10mM TrisHCl buffer pH 8.0. Polypeptides were eluted with a linear gradient of NaCl from 0 to 1.0 M in the above buffer system. The column eluent was monitored at a wavelength of 280 nm.

The pool of polypeptides eluting from the ion exchange column were fractionated by preparative 2D gel electrophoresis. Samples containing 200-500 μg of polypeptide were made 8M in urea and applied to polyacrylamide isoelectric focusing rod gels (diameter 2mm, length 150 mm, pH 5-7). After the isoelectric focusing step, the first dimension gels were equilibrated with reducing buffer and applied to second dimension gels (16% polyacrylamide). Polypeptides from the second dimension separation were transferred to PVDF membranes by electroblotting in 10mM CAPS buffer pH 11 containing 10% (v/v) methanol. The PVDF membranes were stained for protein with Coomassie blue. Regions of PVDF containing polypeptides of interest were cut out and directly introduced into the sample cartridge of the Perkin Elmer/Applied BioSystems Procise 492 protein sequencer. The polypeptides were sequenced from the amino terminal end using traditional Edman chemistry. The amino acid sequence was determined for each polypeptide by comparing the retention time of the PTH amino acid derivative to the appropriate PTH derivative standards. Using these procedures, eleven polypeptides, designated GVs-1, GVs-3, GVs-4, GVs-5, GVs-6, GVs-8, GVs-9, GVs-10, GVs-11, GV-34 and GV-35 were isolated. The determined N-terminal sequences for these polypeptides are shown in SEQ ID NOS: 21-29, 63 and 64, respectively. Using the purification procedure described above, more protein was purified to extend the amino acid sequence previously obtained for GVs-9. The extended amino acid sequence for GVs-9 is provided in SEQ ID NO: 65. Further studies resulted in the isolation

of DNA sequences for GVs-9 (SEQ ID NO: 111) and GV-35 (SEQ ID NO: 155). The corresponding predicted amino acid sequences are provided in SEQ ID NO: 112 and 156, respectively. An extended DNA sequence for GVs-9 is provided in SEQ ID NO: 153, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 154. The predicted amino acid sequence for GVs-9 has been amended in SEQ ID NO: 197.

All of these amino acid sequences were compared to known amino acid sequences in the SwissProt data base (version R35 plus update). No significant homologies were obtained, with the exceptions of GVs-3, GVs-4, GVs-5 and GVs-9. GVs-9 was found to bear some homology to two previously identified *M. tuberculosis* proteins, namely *M. tuberculosis* cutinase precursor and an *M. tuberculosis* hypothetical 22.6 kDa protein. GVs-3, GVs-4 and GVs-5 were found to bear some similarity to the antigen 85A and 85B proteins from *M. leprae* (SEQ ID NOS: 30 and 31, respectively), *M. tuberculosis* (SEQ ID NOS: 32 and 33, respectively) and *M. bovis* (SEQ ID NOS: 34 and 35, respectively), and the antigen 85C proteins from *M. leprae* (SEQ ID NO: 36) and *M. tuberculosis* (SEQ ID NO: 37).

EXAMPLE 12

DNA CLONING STRATEGY FOR THE *M. VACCAE* ANTIGEN 85 SERIES

Probes for antigens 85A, 85B, and 85C were prepared by polymerase chain reaction (PCR) using degenerate oligonucleotides (SEQ ID NOS: 38 and 39) designed to regions of antigen 85 genomic sequence that are conserved between family members in a given mycobacterial species, and between mycobacterial species. These oligonucleotides were used under reduced stringency conditions to amplify target sequences from *M. vaccae* genomic DNA. An appropriately-sized 485 bp band was identified, purified, and cloned into T-tailed pBluescript II SK (Stratagene, La Jolla, CA). Twenty-four individual colonies were screened at random for the presence of the antigen 85 PCR product, then sequenced using the Perkin Elmer/Applied Biosystems Model 377 automated sequencer and the M13-based primers, T3 and T7. Homology searches of the GenBank databases showed that twenty-three clones contained insert with significant homology to published antigen 85 genes from *M.*

tuberculosis and *M. bovis*. Approximately half were most homologous to antigen 85C gene sequences, with the remainder being more similar to antigen 85B sequences. In addition, these two putative *M. vaccae* antigen 85 genomic sequences were 80% homologous to one another. Because of this high similarity, the antigen 85C PCR fragment was chosen to screen *M. vaccae* genomic libraries at low stringency for all three antigen 85 genes.

An *M. vaccae* genomic library was created in lambda Zap-Express (Stratagene, La Jolla, CA) by cloning *Bam*HI partially-digested *M. vaccae* genomic DNA into similarly-digested λ vector, with 3.4 x 10^5 independent plaque-forming units resulting. For screening purposes, twenty-seven thousand plaques from this non-amplified library were plated at low density onto eight 100 cm^2 plates. For each plate, duplicate plaque lifts were taken onto Hybond-N^+ nylon membrane (Amersham International, United Kingdom), and hybridised under reduced-stringency conditions (55 °C) to the radiolabelled antigen 85C PCR product. Autoradiography demonstrated that seventy-nine plaques consistently hybridised to the antigen 85C probe under these conditions. Thirteen positively-hybridising plaques were selected at random for further analysis and removed from the library plates, with each positive clone being used to generate secondary screening plates containing about two hundred plaques. Duplicate lifts of each plate were taken using Hybond-N^+ nylon membrane, and hybridised under the conditions used in primary screening. Multiple positively-hybridising plaques were identified on each of the thirteen plates screened. Two well-isolated positive phage from each secondary plate were picked for further analysis. Using *in vitro* excision, twenty-six plaques were converted into phagemid, and restriction-mapped. It was possible to group clones into four classes on the basis of this mapping. Sequence data from the 5' and 3' ends of inserts from several representatives of each group was obtained using the Perkin Elmer/Applied Biosystems Model 377 automated sequencer and the T3 and T7 primers. Sequence homologies were determined using BLASTN analysis of the EMBL database. Two of these sets of clones were found to be homologous to *M. bovis* and *M. tuberculosis* antigen 85A genes, each containing either the 5' or 3' ends of the *M. vaccae* gene (this gene was cleaved during library construction as it contains an internal *Bam*HI site). The remaining clones were found to contain sequences homologous to antigens 85B and 85C from a number

of mycobacterial species. To determine the remaining nucleotide sequence for each gene, appropriate subclones were constructed and sequenced. Overlapping sequences were aligned using the DNA Strider software. The determined DNA sequences for *M. vaccae* antigens 85A, 85B and 85C are shown in SEQ ID NOS: 40-42, respectively, with the predicted amino acid sequences being shown in SEQ ID NOS: 43-45, respectively.

The *M. vaccae* antigens GVs-3 and GVs-5 were expressed and purified as follows. Amplification primers were designed from the insert sequences of GVs-3 and GVs-5 (SEQ ID NO: 40 and 42, respectively) using sequence data downstream from the putative leader sequence and the 3' end of the clone. The sequences of the primers for GVs-3 are provided in SEQ ID NO: 66 and 67, and the sequences of the primers for GVs-5 are provided in SEQ ID NO: 68 and 69. A *Xho*I restriction site was added to the primers for GVs-3, and *Eco*RI and *Bam*HI restriction sites were added to the primers for GVs-5 for cloning convenience. Following amplification from genomic *M. vaccae* DNA, fragments were cloned into the appropriate site of pProEX HT prokaryotic expression vector (Gibco BRL, Life Technologies, Gaithersburg, MD) and submitted for sequencing to confirm the correct reading frame and orientation. Expression and purification of the recombinant protein was performed according to the manufacturer's protocol.

Expression of a fragment of the *M. vaccae* antigen GVs-4 (antigen 85B homolog) was performed as follows. The primers AD58 and AD59, described above, were used to amplify a 485 bp fragment from *M. vaccae* genomic DNA. This fragment was gel-purified using standard techniques and cloned into *Eco*RV-digested pBluescript containing added dTTP residues. The base sequences of inserts from five clones were determined and found to be identical to each other. These inserts had highest homology to Ag85B from *M. tuberculosis*. The insert from one of the clones was subcloned into the *Eco*RI/*Xho*I sites of pProEX HT prokaryotic expression vector (Gibco BRL), expressed and purified according to the manufacturer's protocol. This clone was renamed GV-4P because only a part of the gene was expressed. The amino acid and DNA sequences for the partial clone GV-4P are provided in SEQ ID NO: 70 and 106, respectively.

Similar to the cloning of GV-4P, the amplification primers AD58 and AD59 were used to amplify a 485 bp fragment from a clone containing GVs-5 (SEQ ID NO:42). This fragment was cloned into the expression vector pET16 and was called GV-5P. The determined nucleotide sequence and predicted amino acid sequence of GV-5P are provided in SEQ ID NOS: 157 and 158, respectively.

In subsequent studies, using procedures similar to those described above, GVs-3, GV-4P and GVs-5 were re-cloned into the alternative vector pET16 (Novagen, Madison, WI).

The ability of purified recombinant GVs-3, GV-4P and GVs-5 to stimulate proliferation of T cells and interferon-γ production in human PBL from PPD-positive, healthy donors, was assayed as described above. The results of this assay are shown in Table 17, wherein (-) indicates a lack of activity, (+/-) indicates polypeptides having a result less than twice higher than background activity of control media, (+) indicates polypeptides having activity two to four times above background, (++) indicates polypeptides having activity greater than four times above background, and ND indicates not determined.

Table 17

	Donor G97005		Donor G97006		Donor G97007		Donor G97008		Donor G97009		Donor G97010	
	Prolif	IFN -γ	Prolif	IFN -γ	Prolif	IFN -γ	Prolif	IFN -γ	Prolif	IFN -γ	Prolif	IFN -γ
GVs-3	++	+	ND	ND	++	++	++	++	++	+/-	+	++
GV - 4P	+	+/-	ND	ND	+	++	++	++	+/-	+/-	+/-	++
GVs-5	++	++	++	++	++	++	+	++	++	+	+	++

EXAMPLE 13

DNA CLONING STRATEGY FOR *M. VACCAE* ANTIGENS

An 84 bp probe for the *M. vaccae* antigen GVc-7 was amplified using degenerate oligonucleotides designed to the determined amino acid sequence of GVc-7 (SEQ ID NOS: 5-8). This probe was used to screen a *M. vaccae* genomic DNA library as described in Example

12. The determined nucleotide sequence for GVc-7 is shown in SEQ ID NO: 46 and predicted amino acid sequence in SEQ ID NO: 47. Comparison of these sequences with those in the databank revealed homology to a hypothetical 15.8 kDa membrane protein of *M. tuberculosis*.

The sequence of SEQ ID NO: 46 was used to design amplification primers (provided in SEQ ID NO: 71 and 72) for expression cloning of the GVc-7 gene using sequence data downstream from the putative leader sequence. A *Xho*I restriction site was added to the primers for cloning convenience. Following amplification from genomic *M. vaccae* DNA, fragments were cloned into the *Xho*I-site of pProEX HT prokaryotic expression vector (Gibco BRL) and submitted for sequencing to confirm the correct reading frame and orientation. Expression and purification of the fusion protein was performed according to the manufacturer's protocol. In subsequent studies, GVc-7 was re-cloned into the vector pET16 (Novagen).

The ability of purified recombinant GVc-7 to stimulate proliferation of T-cells and stimulation of interferon-γ production in human PBL, from PPD-positive, healthy donors, was assayed as described above. The results are shown in Table 18, wherein (-) indicates a lack of activity, (+/-) indicates polypeptides having a result less than twice higher than background activity of control media, (+) indicates polypeptides having activity two to four times above background, and (++) indicates polypeptides having activity greater than four times above background.

TABLE 18

Donor	Proliferation	Interferon-γ
G97005	++	+/-
G97008	++	+
G97009	+	+/-
G97010	+/-	++

A redundant oligonucleotide probe (SEQ ID NO 73; referred to as MPG15) was designed to the GVs-8 peptide sequence shown in SEQ ID NO: 26 and used to screen a *M. vaccae* genomic DNA library using standard protocols. Two genomic clones containing genes encoding four different antigens was isolated. The determined DNA sequences for

GVs-8A (re-named GV-30), GVs-8B (re-named GV-31), GVs-8C (re-named GV-32) and GVs-8D (re-named GV-33) are shown in SEQ ID NOS: 48-51, respectively, with the corresponding amino acid sequences being shown in SEQ ID NOS: 52-55, respectively. GV-30 contains regions showing some similarity to known prokaryotic valyl-tRNA synthetases; GV-31 shows some similarity to *M. smegmatis* aspartate semialdehyde dehydrogenase; and GV-32 shows some similarity to the *H. influenza* folylpolyglutamate synthase gene. GV-33 contains an open reading frame which shows some similarity to sequences previously identified in *M. tuberculosis* and *M. leprae,* but whose function has not been identified.

The determined partial DNA sequence for GV-33 is provided in SEQ ID NO: 74 with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 75. Sequence data from the 3' end of the clone showed homology to a previously identified 40.6 kDa outer membrane protein of *M. tuberculosis*. Subsequent studies led to the isolation of a full-length DNA sequence for GV-33 (SEQ ID NO: 193). The corresponding predicted amino acid sequence is provided in SEQ ID NO: 194.

The gene encoding GV-33 was amplified from *M. vaccae* genomic DNA with primers based on the determined nucleotide sequence. This DNA fragment was cloned into *Eco*Rv-digested pBluescript II SK^+ (Stratagene), and then transferred to pET16 expression vector. Recombinant protein was purified following the manufacturer's protocol.

The ability of purified recombinant GV-33 to stimulate proliferation of T-cells and stimulation of interferon-γ production in human PBL was assayed as described above. The results are shown in Table 19, wherein (-) indicates a lack of activity, (+/-) indicates polypeptides having a result less than twice higher than background activity of control media, (+) indicates polypeptides having activity two to four times above background, and (++) indicates polypeptides having activity greater than four times above background.

TABLE 19

Stimulatory Activity of Polypeptides

Donor	Proliferation	Interferon-γ
G97005	++	+
G97006	++	++
G97007	-	+/-
G97008	+/-	-
G97009	+/-	-
G97010	+/-	++

EXAMPLE 14

ISOLATION OF PROTEINS FROM DD-*M. VACCAE*

M. vaccae bacteria were cultured, pelleted and autoclaved as described in Example 1. Culture filtrates of live *M. vaccae* refer to the supernatant from 24 hour cultures of *M. vaccae* in 7H9 medium with glucose. A delipidated form of *M. vaccae* was prepared by sonicating autoclaved *M. vaccae* for four bursts of 30 seconds on ice using the Virsonic sonicator (Virtis, Disa, USA). The material was then centrifuged (9000 rpm, 20 minutes, JA10 rotor, brake = 5). The resulting pellet was suspended in 100 ml of chloroform/methanol (2:1), incubated at room temperature for 1 hour, re-centrifuged, and the chloroform/methanol extraction repeated. The pellet was obtained by centrifugation, dried *in vacuo*, weighed and resuspended in PBS at 50 mg (dry weight) per ml as delipidated *M. vaccae.*

Glycolipids were removed from the delipidated *M. vaccae* preparation by refluxing in 50% v/v ethanol for 2 hours. The insoluble material was collected by centrifugation (10,000 rpm, JA20 rotor, 15 mins, brake = 5). The extraction with 50% v/v ethanol under reflux was repeated twice more. The insoluble material was collected by centrifugation and washed in PBS. Proteins were extracted by resuspending the pellet in 2% SDS in PBS at 56 °C for 2 hours. The insoluble material was collected by centrifugation and the extraction with 2% SDS/PBS at 56 °C was repeated twice more. The pooled SDS extracts were cooled to 4 °C, and precipitated SDS was removed by centrifugation (10,000 rpm, JA20 rotor, 15 mins, brake

= 5). Proteins were precipitated from the supernatant by adding an equal volume of acetone and incubating at -20 °C for 2 hours. The precipitated proteins were collected by centrifugation, washed in 50% v/v acetone, dried *in vacuo*, and redissolved in PBS.

The SDS-extracted proteins derived from DD-*M. vaccae* were analysed by polyacrylamide gel electrophoresis. Three major bands were observed after staining with silver. In subsequent experiments, larger amounts of SDS-extracted proteins from DD-*M.vaccae*, were analysed by polyacrylamide gel electrophoresis. The proteins, on staining with Coomassie blue, showed several bands. A protein represented by a band of approximate molecular weight of 30 kDa was designated GV-45. The determined N-terminal sequence for GV-45 is provided in SEQ ID NO: 187. A protein of approximate molecular weight of 14 kDa was designated GV-46. The determined N-terminal amino acid sequence of GV-46 is provided in SEQ ID NO: 208.

In subsequent studies, more of the SDS-extracted proteins described above were prepared by preparative SDS-PAGE on a BioRad Prep Cell (Hercules, CA). Fractions corresponding to molecular weight ranges were precipitated by trichloroacetic acid to remove SDS before assaying for adjuvant activity in the anti-ovalbumin-specific cytotoxic response assay in C57BL/6 mice as described above. The adjuvant activity was highest in the 60-70 kDa fraction. The most abundant protein in this size range was purified by SDS-PAGE blotted on to a polyvinylidene difluoride (PVDF) membrane and then sequenced. The sequence of the first ten amino acid residues is provided in SEQ ID NO:76. Comparison of this sequence with those in the gene bank as described above, revealed homology to the heat shock protein 65 (GroEL) gene from *M. tuberculosis*, indicating that this protein is an *M. vaccae* member of the GroEL family.

An expression library of *M. vaccae* genomic DNA in *Bam*H1-lambda ZAP-Express (Stratagene) was screened using sera from cynomolgous monkeys immunised with *M. vaccae* secreted proteins prepared as described above. Positive plaques were identified using a colorimetric system. These plaques were re-screened until plaques were pure following standard procedures. pBK-CMV phagemid 2-1 containing an insert was excised from the lambda ZAP Express (Stratagene) vector in the presence of ExAssist helper phage following

the manufacturer's protocol. The base sequence of the 5' end of the insert of this clone, hereinafter referred to as GV-27, was determined using Sanger sequencing with fluorescent primers on Perkin Elmer/Applied Biosystems Division automatic sequencer. The determined nucleotide sequence of the partial *M. vaccae* GroEL-homologue clone GV-27 is provided in SEQ ID NO: 77 and the predicted amino acid sequence in SEQ ID NO: 78. This clone was found to have homology to *M. tuberculosis* GroEL. A partial sequence of the 65 kDa heat shock protein of *M. vaccae* has been published by Kapur et al. (*Arch. Pathol. Lab. Med. 119* :131-138, 1995). The nucleotide sequence of the Kapur et al. fragment is shown in SEQ ID NO: 79 and the predicted amino acid sequence in SEQ ID NO: 80.

In subsequent studies, an extended (full-length except for the predicted 51 terminal nucleotides) DNA sequence for GV-27 was obtained (SEQ ID NO: 113). The corresponding predicted amino acid sequence is provided in SEQ ID NO: 114. Further studies led to the isolation of a full-length DNA sequence for GV-27 (SEQ ID NO: 159). The corresponding predicted amino acid sequence is provided in SEQ ID NO: 160. GV-27 was found to be 93.7% identical to the *M. tuberculosis* GroEL at the amino acid level.

Two peptide fragments, comprising the N-terminal sequence (hereinafter referred to as GV-27A) and the carboxy terminal sequence of GV-27 (hereinafter referred to as GV-27B) were prepared using techniques well known in the art. The nucleotide sequences for GV-27A and GV-27B are provided in SEQ ID NO: 115 and 116, respectively, with the corresponding amino acid sequences being provided in SEQ ID NO: 117 and 118. Subsequent studies led to the isolation of an extended DNA sequence for GV-27B. This sequence is provided in SEQ ID NO: 161, with the corresponding amino acid sequence being provided in SEQ ID NO: 162. The sequence of GV-27A is 95.8% identical to the *M. tuberculosis* GroEL sequence and contains the shorter *M. vaccae* sequence of Kapur et al. discussed above. The sequence for GV-27B shows about 92.2% identity to the corresponding region of *M. tuberculosis* HSP65. Following the same protocol as for the isolation of GV-27, pBK-CMV phagemid 3-1 was isolated. The antigen encoded by this DNA was named GV-29. The determined nucleotide sequences of the 5' and 3' ends of the gene are provided in SEQ ID NOS: 163 and 164, respectively, with the predicted corresponding amino acid sequences being provided in SEQ

ID NOS: 165 and 166 respectively. GV-29 showed homology to yeast urea amidolyase. The determined DNA sequence for the full-length gene encoding GV-29 is provided in SEQ ID NO: 198, with the corresponding predicted amino acid sequence in SEQ ID NO: 199. The DNA encoding GV-29 was sub-cloned into the vector pET16 (Novagen, Madison, WI) for expression and purification according to standard protocols.

EXAMPLE 15

DNA CLONING STRATEGY FOR THE *M. VACCAE* ANTIGENS GV-23, GV-24, GV-25, GV-26, GV-38A AND GV-38B

M. vaccae (ATCC Number 15483) was grown in sterile Medium 90 at 37 °C for 4 days and harvested by centrifugation. Cells were resuspended in 1 ml Trizol (Gibco BRL, Life Technologies, Gaithersburg, Maryland) and RNA extracted according to the standard manufacturer's protocol. *M. tuberculosis* strain H37Rv (ATCC Number 27294) was grown in sterile Middlebrook 7H9 medium with Tween 80™ and oleic acid/ albumin/dextrose/catalase additive (Difco Laboratories, Detroit, Michigan) at 37 °C and harvested under appropriate laboratory safety conditions. Cells were resuspended in 1 ml Trizol (Gibco BRL) and RNA extracted according to the manufacturer's standard protocol.

Total *M. tuberculosis* and *M. vaccae* RNA was depleted of 16S and 23S ribosomal RNA (rRNA) by hybridisation of the total RNA fraction to oligonucleotides AD10 and AD11 (SEQ ID NO: 81 and 82) complementary to *M. tuberculosis* rRNA. These oligonucleotides were designed from mycobacterial 16S rRNA sequences published by Bottger (*FEMS Microbiol. Lett. 65*:171-176, 1989) and from sequences deposited in the databanks. Depletion was done by hybridisation of total RNA to oligonucleotides AD10 and AD11 immobilised on nylon membranes (Hybond N, Amersham International, United Kingdom). Hybridisation was repeated until rRNA bands were not visible on ethidium bromide-stained agarose gels. An oligonucleotide, AD12 (SEQ ID NO: 83), consisting of 20 dATP-residues, was ligated to the 3' ends of the enriched mRNA fraction using RNA ligase. First strand cDNA synthesis was performed following standard protocols, using oligonucleotide AD7 (SEQ ID NO:84) containing a poly(dT) sequence.

The *M. tuberculosis* and *M. vaccae* cDNA was used as template for single-sided-specific PCR (3S-PCR). For this protocol, a degenerate oligonucleotide AD1 (SEQ ID NO:85) was designed based on conserved leader sequences and membrane protein sequences. After 30 cycles of amplification using primer AD1 as 5'-primer and AD7 as 3'-primer, products were separated on a urea/polyacrylamide gel. DNA bands unique to *M. vaccae* were excised and re-amplified using primers AD1 and AD7. After gel purification, bands were cloned into pGEM-T (Promega) and the base sequence determined.

Searches with the determined nucleotide and predicted amino acid sequences of band 12B21 (SEQ ID NOS: 86 and 87, respectively) showed homology to the *pota* gene of *E.coli* encoding the ATP-binding protein of the spermidine/putrescine ABC transporter complex published by Furuchi et al. (*Jnl. Biol. Chem. 266*: 20928-20933, 1991). The spermidine/putrescine transporter complex of *E.coli* consists of four genes and is a member of the ABC transporter family. The ABC (ATP-binding Cassette) transporters typically consist of four genes: an ATP-binding gene, a periplasmic, or substrate binding, gene and two transmembrane genes. The transmembrane genes encode proteins each characteristically having six membrane-spanning regions. Homologues (by similarity) of this ABC transporter have been identified in the genomes of *Haemophilus influenza* (Fleischmann et al. *Science 269* :496-512, 1995) and *Mycoplasma genitalium* (Fraser, et al. *Science*, *270*:397-403, 1995).

An *M. vaccae* genomic DNA library constructed in BamH1-digested lambda ZAP Express (Stratagene) was probed with the radiolabelled 238 bp band 12B21 following standard protocols. A plaque was purified to purity by repetitive screening and a phagemid containing a 4.5 kb insert was identified by Southern blotting and hybridisation. The nucleotide sequence of the full-length *M. vaccae* homologue of *pota* (ATP-binding protein) was identified by subcloning of the 4.5 kb fragment and base sequencing. The gene consisted of 1449 bp including an untranslated 5' region of 320 bp containing putative -10 and -35 promoter elements. The nucleotide and predicted amino acid sequences of the *M. vaccae pota* homologue are provided in SEQ ID NO: 88 and 89, respectively.

The nucleotide sequence of the *M. vaccae pota* gene was used to design primers EV24 and EV25 (SEQ ID NO: 90 and 91) for expression cloning. The amplified DNA fragment

was cloned into pProEX HT prokaryotic expression system (Gibco BRL) and expression in an appropriate *E.coli* host was induced by addition of 0.6 mM isopropylthio-β-galactoside (IPTG). The recombinant protein was named GV-23 and purified from inclusion bodies according to the manufacturer's protocol. In subsequent studies, GV-23 (SEQ ID NO: 88) was re-cloned into the alternative vector pET16 (Novagen). The amino acid sequence of SEQ ID NO: 89 contains an ATP binding site at residues 34 to 41. At residues 116 to 163 of SEQ ID NO: 89, there is a conserved region that is found in the ATP-transporter family of proteins. These findings suggest that GV-23 is an ATP binding protein.

A 322 bp *Sal*1-*Bam*H1 subclone at the 3'-end of the 4.5 kb insert described above showed homology to the *potd* gene, (periplasmic protein), of the spermidine/putrescine ABC transporter complex of *E. coli.* The nucleotide sequence of this subclone is shown in SEQ ID NO:92. To identify the gene, the radiolabelled insert of this subclone was used to probe a *M. vaccae* genomic DNA library constructed in the *Sal*1-site of lambda Zap Express (Stratagene) following standard protocols. A clone was identified of which 1342 bp showed homology with the *potd* gene of *E. coli.* The *potd* homologue of *M. vaccae* was identified by sub-cloning and base sequencing. The determined nucleotide and predicted amino acid sequences are shown in SEQ ID NO: 93 and 94.

For expression cloning, primers EV-26 and EV-27 (SEQ ID NOS: 95-96) were designed from the determined *M. vaccae potd* homologue. The amplified fragment was cloned into pProEX HT Prokaryotic expression system (Gibco BRL). Expression in an appropriate *E. coli* host was induced by addition of 0.6 mM IPTG and the recombinant protein named GV-24. The recombinant antigen was purified from inclusion bodies according to the protocol of the supplier. In subsequent studies, GV-24 (SEQ ID NO: 93) was re-cloned into the alternative vector pET16 (Novagen).

To improve the solubility of the purified recombinant antigen, the gene encoding GV-24, but excluding the signal peptide, was re-cloned into the expression vector, employing. amplification primers EV101 and EV102 (SEQ ID NOS: 167 and 168). The construct was designated GV-24B. The nucleotide sequence of GV-24B is provided in SEQ ID NO: 169

and the predicted amino acid sequence in SEQ ID NO: 170. This fragment was cloned into pET16 for expression and purification of GV-24B according to the manufacturer's protocols.

The ability of purified recombinant protein GV-23 and GV-24 to stimulate proliferation of T cells and interferon-γ production in human PBL was determined as described above. The results of these assays are provided in Table 20, wherein (-) indicates a lack of activity, (+/-) indicates polypeptides having a result less than twice higher than background activity of control media, (+) indicates polypeptides having activity two to four times above background, (++) indicates polypeptides having activity greater than four times above background, and (ND) indicates not determined.

TABLE 20

	Donor G97005		Donor G97006		Donor G97007		Donor G97008		Donor G97009		Donor G97010	
	Prolif	IFN-γ	Prolif	IFN-γ	Prolif	IFN-γ	Prolif	IFN-γ	Prolif	IFN-γ	Prolif	IFN-γ
GV-23	++	++	++	++	+	+	++	++	+	-	+	++
GV-24	++	+	++	+	ND	ND	+	+/-	+	+/-	+/-	++

Base sequence adjacent to the *M. vaccae potd* gene-homologue was found to show homology to the *potb* gene of the spermidine/putrescine ABC transporter complex of *E.coli*, which is one of two transmembrane proteins in the ABC transporter complex. The *M. vaccae potb* homologue (referred to as GV-25) was identified through further subcloning and base sequencing. The determined nucleotide and predicted amino acid sequences for GV-25 are shown in SEQ ID NOS: 97 and 98, respectively.

Further subcloning and base sequence analysis of the adjacent 509 bp failed to reveal significant homology to PotC, the second transmembrane protein of *E.coli*, and suggests that a second transmembrane protein is absent in the *M. vaccae* homologue of the ABC transporter. An open reading frame with homology to *M. tuberculosis* acetyl-CoA acetyl transferase, however, was identified starting 530 bp downstream of the transmembrane protein and the translated protein was named GV-26. The determined partial nucleotide sequence and predicted amino acid sequence for GV-26 are shown in SEQ ID NO: 99 and 100, respectively.

Using a protocol similar to that described above for the isolation of GV-23, the 3S-PCR band 12B28 (SEQ ID NO: 119) was used to screen the *M. vaccae* genomic library constructed in the BamHI-site of lambda ZAP Express (Stratagene). The clone isolated from the library contained a novel open reading frame and the antigen encoded by this gene was named GV-38A. The determined nucleotide sequence and predicted amino acid sequence of GV-38A are shown in SEQ ID NO: 120 and 121, respectively. Subsequent studies led to the isolation of an extended DNA sequence for GV-38A, provided in SEQ ID NO: 171. The corresponding amino acid sequence is provided in SEQ ID NO: 172. Comparison of these sequences with those in the gene bank, revealed some homology to an unknown *M. tuberculosis* protein previously identified in cosmid MTCY428.12. (SPTREMBL:P71915).

Upstream of the GV-38A gene, a second novel open reading frame was identified and the antigen encoded by this gene was named GV-38B. The determined 5' and 3' nucleotide sequences for GV-38B are provided in SEQ ID NO: 122 and 123, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 124 and 125, respectively. Further studies led to the isolation of the full-length DNA sequence for GV-38B, provided in SEQ ID NO: 173. The corresponding amino acid sequence is provided in SEQ ID NO: 174. This protein was found to show homology to an unknown *M. tuberculosis* protein identified in cosmid MTCY428.11 (SPTREMBL: P71914).

Both the GV-38A and GV-38B antigens were amplified for expression cloning into pET16 (Novagen). GV-38A was amplified with primers KR11 and KR12 (SEQ ID NO: 126 and 127) and GV-38B with primers KR13 and KR14 (SEQ ID NO: 128 and 129). Protein expression in the host cells BL21(DE3) was induced with 1 mM IPTG, however no protein expression was obtained from these constructs. Hydrophobic regions were identified in the N-termini of antigens GV-38A and GV-38B which may inhibit expression of these constructs. The hydrophobic region present in GV-38A was identified as a possible transmembrane motif with six membrane spanning regions. To express the antigens without the hydrophobic regions, primers KR20 for GV-38A, (SEQ ID NO: 130) and KR21 for GV-38B (SEQ ID NO: 131) were designed. The truncated GV-38A gene was amplified with primers KR20 and KR12, and the truncated GV-38B gene with KR21 and KR14. The determined nucleotide

sequences of truncated GV38A and GV-38B are shown in SEQ ID NO: 132 and 133, respectively, with the corresponding predicted amino acid sequences being shown in SEQ ID NO: 134 and 135, respectively. Extended DNA sequences for truncated GV-38A and GV-38B are provided in SEQ ID NO: 175 and 176, respectively, with the corresponding amino acid sequences being provided in SEQ ID NO: 177 and 178, respectively.

EXAMPLE 16

PURIFICATION AND CHARACTERISATION OF POLYPEPTIDES FROM *M. VACCAE* CULTURE FILTRATE BY PREPARATIVE ISOELECTRIC FOCUSING AND PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

M. vaccae soluble proteins were isolated from culture filtrate using preparative isoelectric focusing and preparative polyacrylamide gel electrophoresis as described below. Unless otherwise noted, all percentages in the following example are weight per volume.

M. vaccae (ATCC Number 15483) was cultured in 250 l sterile Medium 90 which had been fractionated by ultrafiltration to remove all proteins of greater than 10 kDa molecular weight. The medium was centrifuged to remove the bacteria, and sterilised by filtration through a 0.45 μm filter. The sterile filtrate was concentrated by ultrafiltration over a 10 kDa molecular weight cut-off membrane.

Proteins were isolated from the concentrated culture filtrate by precipitation with 10% trichloroacetic acid. The precipitated proteins were re-dissolved in 100 mM Tris.HCl pH 8.0. and re-precipitated by the addition of an equal volume of acetone. The acetone precipitate was dissolved in water, and proteins were re-precipitated by the addition of an equal volume of chloroform:methanol 2:1 (v/v). The chloroform:methanol precipitate was dissolved in water, and the solution was freeze-dried.

The freeze-dried protein was dissolved in iso-electric focusing buffer, containing 8 M deionised urea, 2% Triton X-100, 10 mM dithiothreitol and 2% ampholytes (pH 2.5 - 5.0). The sample was fractionated by preparative iso-electric focusing on a horizontal bed of Ultrodex gel at 8 watts constant power for 16 hours. Proteins were eluted from the gel bed fractions with water and concentrated by precipitation with 10% trichloroacetic acid.

Pools of fractions containing proteins of interest were identified by analytical polyacrylamide gel electrophoresis and fractionated by preparative polyacrylamide gel electrophoresis. Samples were fractionated on 12.5% SDS-PAGE gels, and electroblotted onto nitrocellulose membranes. Proteins were located on the membranes by staining with Ponceau Red, destained with water and eluted from the membranes with 40% acetonitrile/0.1M ammonium bicarbonate pH 8.9 and then concentrated by lyophilisation.

Eluted proteins were assayed for their ability to induce proliferation and interferon-γ secretion from the peripheral blood lymphocytes of immune donors as detailed above. Proteins inducing a strong response in these assays were selected for further study.

Selected proteins were further purified by reversed-phase chromatography on a Vydac Protein C4 column, using a trifluoroacetic acid-acetonitrile system. Purified proteins were prepared for protein sequence determination by SDS-polyacrylamide gel electrophoresis, and electroblotted onto PVDF membranes. Protein sequences were determined as in Example 3. The proteins were named GV-40, GV-41, GV-42, GV-43 and GV-44. The determined N-terminal sequences for these polypeptides are shown in SEQ ID NOS: 101-105, respectively. Subsequent studies led to the isolation of a 5', middle fragment and 3' DNA sequence for GV-42 (SEQ ID NO: 136, 137 and 138, respectively). The corresponding predicted amino acid sequences are provided in SEQ ID NO: 139, 140 and 141, respectively.

Following standard DNA amplification and cloning procedures as described in Example 13, the genes encoding GV-41 and GV-42 were cloned. The determined nucleotide sequences are provided in SEQ ID NOS: 179 and 180, respectively, and the predicted amino acid sequences in SEQ ID NOS: 181 and 182. Further experiments lead to the cloning of the full-length gene encoding GV-41, which was named GV-41B. The determined nucleotide sequence and the predicted amino acid sequence of GV-41B are provided in SEQ ID NOS: 202 and 203, respectively. GV-41 had homology to the ribosome recycling factor of *M. tuberculosis* and *M. leprae*, and GV-42 had homology to a *M. avium* fibronectin attachment protein FAP-A. Within the full-length sequence of GV-42, the amino acid sequence determined for GV-43 (SEQ ID NO: 104) was identified, indicating that the amino acid sequences for GV-42 and GV-43 were obtained from the same protein.

Murine polyclonal antisera were prepared against GV-40 and GV-44 following standard procedures. These antisera were used to screen a *M. vaccae* genomic DNA library consisting of randomly sheared DNA fragments. Clones encoding GV-40 and GV-44 were identified and sequenced. The determined nucleotide sequence of the partial gene encoding GV-40 is provided in SEQ ID NO: 183 and the predicted amino acid sequence in SEQ ID NO:184. The complete gene encoding GV-40 was not cloned, and the antigen encoded by this partial gene was named GV-40P. An extended DNA sequence for GV-40P is provided in SEQ ID NO: 206 with the corresponding predicted amino acid sequence being provided in SEQ ID NO 207. The determined nucleotide sequence of the gene encoding GV-44 is provided in SEQ ID NO: 185, and the predicted amino acid sequence in SEQ ID NO: 186. With further sequencing, the determined DNA sequence for the full-length gene encoding GV-44 was obtained and is provided in SEQ ID NO 204, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 205. Homology of GV-40 to *M. leprae* Elongation factor G was found and GV-44 had homology to *M. leprae* glyceraldehyde-3-phosphate dehydrogenase.

EXAMPLE 17

ISOLATION OF THE DD-*M. VACCAE* ANTIGENS GV-45 AND GV-46

Proteins were extracted from DD-*M. vaccae* (500 mg; prepared as described above) by suspension in 10 ml 2% SDS/PBS and heating to 50 °C for 2 h. The insoluble residue was removed by centrifugation, and proteins precipitated from the supernatant by adding an equal volume of acetone and incubating at –20 °C for 1 hr. The precipitated proteins were collected by centrifugation, dissolved in reducing sample buffer, and fractionated by preparative SDS-polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto PVDF membrane in 10 mM CAPS/0.01% SDS pH 11.0, and N-terminal sequences were determined in a gas-phase sequenator.

From these experiments, a protein represented by a band of approximate molecular weight of 30 kDa, designated GV-45, was isolated. The determined N-terminal sequence for GV-45 is provided in SEQ ID NO: 187. From the same experiments, a protein of

approximate molecular weight of 14 kDa, designated GV-46, was obtained. The determined N-terminal amino acid sequence of GV-46 is provided in SEQ ID NO: 208. GV-46 is homologous to the highly conserved mycobacterial host integration factor of *M. tuberculosis* and *M. smegmatis*.

From the amino acid sequence of GV-45, degenerate oligonucleotides KR32 and KR33 (SEQ ID NOS: 188 and 189, respectively) were designed. A 100 bp fragment was amplified, cloned into plasmid pBluescript II SK^+ (Stratagene, La Jolla, CA) and sequenced (SEQ ID NO:190) following standard procedures (Sambrook, *Ibid*). The cloned insert was used to screen a *M. vaccae* genomic DNA library constructed in the *Bam*HI-site of lambda ZAP-Express (Stratagene). The isolated clone showed homology to a 35 kDa *M. tuberculosis* and a 22 kDa *M. leprae* protein containing bacterial histone-like motifs at the N-terminus and a unique C-terminus consisting of a five amino acid basic repeat. The determined nucleotide sequence for GV-45 is provided in SEQ ID NO: 191, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 192. With additional sequencing, the determined DNA sequence for the full-length gene encoding GV-45 was obtained and is provided in SEQ ID NO: 200, with the corresponding predicted amino acid sequence in SEQ ID NO: 201.

EXAMPLE 18

IMMUNOGENICITY AND IMMUNOMODULATING PROPERTIES OF RECOMBINANT PROTEINS DERIVED FROM *M.VACCAE*

A. INDUCTION OF T CELL PROLIFERATION AND IFN-γ PRODUCTION

The immunogenicity of *Mycobacterium vaccae* recombinant proteins (GV recombinant proteins) was tested by injecting female BALB/cByJ mice in each hind foot-pad with 10 ug of recombinant GV proteins emulsified in incomplete Freund's adjuvant (IFA). Control mice received phosphate buffered saline in IFA. The draining popliteal lymph nodes were excised 10 days later and the cells obtained therefrom were stimulated with the immunizing GV protein and assayed for proliferation by measuring the uptake of tritiated

thymidine. The amount of interferon gamma (IFNγ) produced and secreted by these cells into the culture supernatants was assayed by standard enzyme-linked immunoassay.

As shown in Table 21 summarising proliferative responses, all GV proteins were found to induce a T cell proliferative response. The lymph node T cells from an immunized mouse proliferated in response to the specific GV protein used in the immunization. Lymph node cells from non-immunised mice did not proliferate in response to GV proteins. The data in Table 22 showing IFNγ production, indicate that most of the GV proteins stimulated IFNγ production by lymph node cells from mice immunised with the corresponding GV protein. When lymph node cells from non-immunized mice were cultured with individual GV proteins, IFNγ production was not detectable.

The GV proteins are thus immunogenic in being able to stimulate T cell proliferation and/or IFNγ production when administered by subcutaneous injection. The antigen-specific stimulatory effects on T cell proliferation and IFNγ production are two advantageous properties of candidate vaccines for tuberculosis.

TABLE 21

Immunogenic Properties of GV proteins: Proliferation

GV protein	Proliferation (cpm)		
	Dose of GV protein used *in vitro* (μg/ml)		
	50	2	0.08
GV-1/70	31,550 ± 803	19,058 ± 2,449	5,596 ± 686
GV-1/83	18,549 ± 2,716	23,932 ± 1,964	11,787 ± 1,128
GV-3	34,751 ± 1,382	6,379 ± 319	4,590 ± 1,042
GV-4P	26,460 ± 1,877	10,370 ± 667	6,685 ± 673
GV-5	42,418 ± 2,444	23,902 ± 2,312	13,973 ± 772
GV-5P	35,691 ± 159	14,457 ± 1,185	8,340 ± 725
GV-7	38,686 ± 974	22,074 ± 3,698	15,906 ± 1,687
GV-9	30,599 ± 2580	15,260 ± 2,764	4,531 ± 1,240
GV-13	15,296 ± 2,006	7,163 ± 833	3,701 ± 243
GV-14	27,754 ± 1,872	13,001 ± 3,273	9,897 ± 2,833
GV-14B	10,761 ± 485	5,075 ± 1,470	2,341 ± 289
GV-22B	3,199 ± 771	3,255 ± 386	1,841 ± 318
GV-23	35,598 ± 1,330	15,423 ± 2,858	7,393 ± 2,188
GV-24B	43,678 ± 2,190	30,307 ± 1,533	15,375 ± 2,594
GV-27	18,165 ± 3,300	16,329 ± 1,794	6,107 ± 1,773
GV-27A	23,723 ± 850	6,860 ± 746	4,295 ± 780
GV-27B	31,602 ± 1,939	29,468 ± 3,867	30,306 ± 1,912
GV-29	20,034 ± 3,328	8,107 ± 488	2,982 ± 897
GV-33	41,529 ± 1,919	27,529 ± 1,238	8,764 ± 256
GV-35	29,163 ± 2,693	9,968 ± 314	1,626 ± 406
GV-38AP	28,971 ± 4,499	17,396 ± 878	8,060 ± 810
GV-38BP	19,746 ± 245	11,732 ± 3,207	6,264 ± 875
GV-40P	25,185 ± 2,877	19,292 ± 2,294	10,883 ± 893
GV-41B	24,646 ± 2,714	12,627 ± 3,622	5,772 ± 1,041
GV-42	25,486 ± 3,029	20,591 ± 2,021	13,789 ± 775
GV-44	2,684 ± 1,995	3,577 ± 1,725	1,499 ± 959
GV-45	9,554 ± 482	3,683 ± 1,127	1,497 ± 199

TABLE 22

Immunogenic properties of GV proteins: IFNγ production

GV protein	IFNγ (ng/ml)		
	Dose of GV protein used *in vitro* (μg/ml)		
	50	10	2
GV-1/70	24.39 ± 6.66	6.19 ± 1.42	1.90 ± 0.53
GV-1/83	11.34 ± 5.46	5.36 ± 1.34	2.73 ± 1.55
GV-3	3.46 ± 0.30	1.57 ± 0.04	not detectable
GV-4P	6.48 ± 0.37	3.00 ± 0.52	1.38 ± 0.50
GV-5	4.08 ± 1.41	6.10 ± 2.72	2.35 ± 0.40
GV-5P	34.98 ± 15.26	9.95 ± 3.42	5.68 ± 0.79
GV-7	33.52 ± 3.08	25.47 ± 4.14	9.60 ± 1.74
GV-9	92.27 ± 45.50	88.54 ± 16.48	30.46 ± 1.77
GV-13	11.60 ± 2.89	2.04 ± 0.58	1.46 ± 0.62
GV-14	8.28 ± 1.56	3.19 ± 0.56	0.94 ± 0.24
GV-14B	not detectable	not detectable	not detectable
GV-22B	not detectable	not detectable	not detectable
GV-23	59.67 ± 14.88	30.70 ± 4.48	9.17 ± 1.51
GV-24B	6.76 ± 0.58	3.20 ± 0.50	1.97 ± 0.03
GV-27	72.22 ± 11.14	30.86 ± 10.55	21.38 ± 3.12
GV-27A	4.25 ± 2.32	1.51 ± 0.73	not detectable
GV-27B	87.98 ± 15.78	44.43 ± 8.70	21.49 ± 5.60
GV-29	7.56 ± 2.58	1.22 ± 0.56	not detectable
GV-33	7.71 ± 0.26	8.44 ± 2.35	1.52 ± 0.24
GV-38AP	23.49 ± 5.89	8.87 ± 1.62	4.17 ± 1.72
GV-38BP	5.30 ± 0.95	3.10 ± 1.19	1.91 ± 1.01
GV-40P	15.65 ± 7.89	10.58 ± 1.31	3.57 ± 1.53
GV-41B	16.73 ± 1.61	5.08 ± 1.08	2.13 ± 1.10
GV-42	95.97 ± 23.86	52.88 ± 5.79	30.06 ± 8.94
GV-44	not detectable	not detectable	not detectable

B. ACTIVATION OF LYMPHOCYTE SUBPOPULATIONS

The ability of recombinant *M. vaccae* proteins of the present invention, heat-killed *M. vaccae* and DD-*M. vaccae* to activate lymphocyte subpopulations was determined by examining upregulation of expression of CD69 (a surface protein expressed on activated cells).

PBMC from normal donors (5 x 10^6 cells/ml) were stimulated with 20 ug/ml of either heat-killed *M. vaccae* cells, DD-*M. vaccae* or recombinant GV-22B (SEQ ID NO: 145), GV-23 (SEQ ID NO: 89), GV-27 (SEQ ID NO: 160), GV27A (SEQ ID NO: 117), GV-27B (SEQ ID NO: 162) or GV-45 (SEQ ID NO: 201) for 24 hours. CD69 expression was determined by staining cultured cells with monoclonal antibody against CD56, αβT cells or γδT cells, in combination with monoclonal antibodies against CD69, followed by flow cytometry analysis

Table 23 shows the percentage of αβT cells, γδT cells and NK cells expressing CD69 following stimulation with heat-killed *M. vaccae*, DD-*M. vaccae* or recombinant *M. vaccae* proteins. These results demonstrate that heat-killed *M. vaccae*, DD-*M. vaccae* and GV-23 stimulate the expression of CD69 in the lymphocyte subpopulations tested compared with control (non-stimulated cells), with particularly high levels of CD69 expression being seen in NK cells. GV-45 was found to upregulate CD69 expression in αβT cells.

TABLE 23
Stimulation of CD69 Expression

	αβT cells	γδT cells	NK cells
Control	3.8	6.2	4.8
Heat-killed *M. vaccae*	8.3	10.2	40.3
DD-*M. vaccae*	10.1	17.5	49.9
GV-22B	5.6	3.9	8.6
GV-23	5.8	10.0	46.8
GV-27	5.5	4.4	13.3
GV-27A	5.5	4.4	13.3
GV-27B	4.4	2.8	7.1
GV-45	11.7	4.9	6.3

The ability of the recombinant protein GV-23 (20 ug/ml) to induce CD69 expression in lymphocyte subpopulations was compared with that of the known Th1-inducing adjuvants MPL/TDM/CWS (Monophosphoryl Lipid A/ Trehalose 6'6' dimycolate; Sigma, St. Louis, MO; at a final dilution of 1:20) and CpG ODN (Promega, Madison, WI; 20 ug/ml), and the known Th2-inducing adjuvants aluminium hydroxide (Superfos Biosector, Kvistgard, Denmark; at a final dilution of 1:400) and cholera toxin (20 ug/ml), using the procedure described above. MPL/TDM/CWS and aluminium hydroxide were employed at the maximum concentration that does not cause cell cytotoxicity. Figs. 8A-C show the stimulation of CD69 expression on αβT cells, γδT cells and NK cells, respectively. GV-23, MPL/TDM/CWS and CpG ODN induced CD69 expression on NK cells, whereas aluminium hydroxide and cholera toxin did not.

C. STIMULATION OF CYTOKINE PRODUCTION

The ability of recombinant *M. vaccae* proteins of the present invention to stimulate cytokine production in PBMC was examined as follows. PBMC from normal donors (5 x 10^6 cells/ml) were stimulated with 20 ug/ml of either heat-killed *M. vaccae* cells, DD-*M. vaccae*, or recombinant GV-22B (SEQ ID NO: 145), GV-23 (SEQ ID NO: 89), GV-27 (SEQ ID NO: 160), GV27A (SEQ ID NO: 117), GV-27B (SEQ ID NO: 162) or GV-45 (SEQ ID NO: 201) for 24 hours. Culture supernatants were harvested and tested for the production of IL-1β, TNF-α, IL-12 and IFN-γ using standard ELISA kits (Genzyme, Cambridge, MA), following the manufacturer's instructions. Figs. 9A-D show the stimulation of IL-1β, TNF-α, IL-12 and IFN-γ production, respectively. Heat-killed *M. vaccae* and DD-*M. vaccae* were found to stimulate the production of all four cytokines examined, while recombinant GV-23 and GV-45 were found to stimulate the production of IL-1β, TNF-α and IL-12. Figs. 10A-C show the stimulation of IL-1β, TNF-α and IL-12 production, respectively, in human PBMC (determined as described above) by varying concentrations of GV-23 and GV-45.

Figs. 11A-D show the stimulation of IL-1β, TNF-α, IL-12 and IFN-γ production, respectively, in PBMC by GV-23 as compared to that by the adjuvants MPL/TDM/CWS (at a final dilution of 1:20), CpG ODN (20 ug/ml), aluminium hydroxide (at a final dilution of 1:400) and cholera toxin (20 ug/ml). GV-23, MPL/TDM/CWS and CpG ODN induced significant levels of the four cytokines examined, with higher levels of IL-1β production being seen with GV-23 than with any of the known adjuvants. Aluminium hydroxide and cholera toxin induced only negligible amounts of the four cytokines.

D. ACTIVATION OF ANTIGEN PRESENTING CELLS

The ability of heat-killed *M. vaccae*, DD-*M. vaccae* and recombinant *M. vaccae* proteins to enhance the expression of the co-stimulatory molecules CD40, CD80 and CD86 on B cells, monocytes and dendritic cells was examined as follows.

Peripheral blood mononuclear cells depleted of T cells and comprising mainly B cells, monocytes and dendritic cells were stimulated with 20 ug/ml of either heat-killed *M. vaccae* cells, DD-*M. vaccae*, or recombinant GV-22B (SEQ ID NO: 145), GV-23 (SEQ ID NO: 89),

GV-27 (SEQ ID NO: 160), GV27A (SEQ ID NO: 117), GV-27B (SEQ ID NO: 162) or GV-45 (SEQ ID NO: 201) for 48 hours. Stimulated cells were harvested and analyzed for up-regulation of CD40, CD80 and CD86 using 3 color flow cytometric analysis. Tables 24, 25 and 26 show the fold increase in mean fluorescence intensity from control (non-stimulated cells) for dendritic cells, monocytes, and B cells, respectively.

TABLE 24

Stimulation of CD40, CD80 and CD86 Expression on Dendritic Cells

	CD40	CD80	CD86
Control	0	0	0
Heat-killed *M. vaccae*	6.1	3.8	1.6
DD-*M. vaccae*	6.6	4.2	1.6
GV-22B	4.6	1.9	1.6
GV-23	6.0	4.5	1.8
GV-27	5.2	1.9	1.6
GV-27A	2.3	0.9	1.0
GV-27B	2.6	1.1	1.1
GV-45	5.8	3.0	3.1

TABLE 25

Stimulation of CD40, CD80 and CD86 Expression on Monocytes

	CD40	CD80	CD86
Control	0	0	0
Heat-killed *M. vaccae*	2.3	1.8	0.7
DD-*M. vaccae*	1.9	1.5	0.7
GV-22B	0.7	0.9	1.1
GV-23	2.3	1.5	0.7
GV-27	1.5	1.4	1.2
GV-27A	1.4	1.4	1.4
GV-27B	1.6	1.2	1.2
GV-45	1.6	1.2	1.0

TABLE 26

Stimulation of CD40, CD80 and CD86 Expression on B Cells

	CD40	CD80	CD86
Control	0	0	0
Heat-killed *M. vaccae*	1.6	1.0	1.7
DD-*M. vaccae*	1.5	0.9	1.7
GV-22B	1.1	0.9	1.2
GV-23	1.2	1.1	1.4
GV-27	1.1	0.9	1.1
GV-27A	1.0	1.1	0.9
GV-27B	1.0	0.9	0.9
GV-45	1.2	1.1	1.3

As shown above, increased levels of CD40, CD80 and CD86 expression were seen in dendritic cells, monocytes and B cells with all the compositions tested. Expression levels were most increased in dendritic cells, with the highest levels of expression being obtained with heat-killed *M. vaccae*, DD-*M. vaccae*, GV-23 and GV-45. Figs. 12A-C show the stimulation of expression of CD40, CD80 and CD86, respectively, in dendritic cells by varying concentrations of GV-23 and GV-45.

The ability of GV-23 to stimulate CD40, CD80 and CD86 expression in dendritic cells was compared to that of the Th1-inducing adjuvants MPL/TDM/CWS (at a final dilution of 1:20) and CpG ODN (20 ug/ml), and the known Th2-inducing adjuvants aluminium hydroxide (at a final dilution of 1:400) and cholera toxin (20 ug/ml). GV23, MPL/TDM/CWS and CpG ODN caused significant up-regulation of CD40, CD80 and CD86, whereas cholera toxin and aluminium hydroxide induced modest or negligible dendritic cell activation, respectively.

E. DENDRITIC CELL MATURATION AND FUNCTION

The effect of the recombinant *M. vaccae* protein GV-23 on the maturation and function of dendritic cells was examined as follows.

Purified dendritic cells ($5 \times 10^4 - 10^5$ cells/ml) were stimulated with GV-23 (20 ug/ml) or LPS (10 ug/ml) as a positive control. Cells were cultured for 20 hour and then analyzed for CD83 (a maturation marker) and CD80 expression by flow cytometry. Non-stimulated cells were used as a negative control. The results are shown below in Table 27.

TABLE 27
Stimulation of CD83 Expression in Dendritic Cells

Treatments	%CD83-positive dendritic cells	% CD80-positive dendritic cells
Control	15 ± 8	9 ± 6.6
GV-23	35 ± 13.2	24.7 ± 14.2
LPS	36.3 ± 14.8	27.7 ± 13

Data = mean ± SD (n=3)

The ability of GV-23 to enhance dendritic cell function as antigen presenting cells was determined by mixed lymphocyte reaction (MLR) assay. Purified dendritic cells were culture in medium alone or with GV-23 (20 ug/ml) for 18-20 hours and then stimulated with allogeneic T cells (2 x 10^5 cells/well). After 3 days of incubation, (^{3}H)-thymidine was added. Cells were harvested 1 day later and the uptake of radioactivity was measured. Fig. 13 shows the increase in uptake of (^{3}H)-thymidine with increase in the ratio of dendritic cells to T cells. Significantly higher levels of radioactivity uptake were seen in GV-23 stimulated dendritic cells compared to non-stimulated cells, showing that GV-23 enhances dendritic cell mixed leukocyte reaction.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, changes and modifications can be carried out without departing from the scope of the invention which is intended to be limited only by the scope of the appended claims.

Claims

1. A polypeptide comprising an immunogenic portion of an isolated *M. vaccae* antigen, wherein the antigen includes a sequence selected from the group consisting of: sequences recited in SEQ ID NOS: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207.

2. A polypeptide comprising an immunogenic portion of an isolated *M. vaccae* antigen, wherein the antigen includes a sequence selected from the group consisting of:

(a) sequences having at least about 50% identical residues to a sequence recited in SEQ ID NOS: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207 as measured by computer algorithm BLASTP;

(b) sequences having at least about 75% identical residues to a sequence recited in SEQ ID NOS: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207 as measured by computer algorithm BLASTP; and

(c) sequences having at least about 95% identical residues to a sequence recited in SEQ ID NOS: 143, 145, 147, 152, 154 156, 158, 160, 162, 165, 166, 170, 172, 174, 177, 178, 181, 182, 184, 186, 187, 192, 194, 196, 197, 199, 201, 203, 205 and 207 as measured by computer algorithm BLASTP.

3. A polypeptide comprising an immunogenic portion of an isolated *M. vaccae* antigen, wherein the antigen comprises an amino acid sequence encoded by a polynucleotide selected from the group consisting of:

(a) sequences recited in SEQ ID NOS: 142, 144, 146, 151, 153, 155, 157, 159, 161, 163, 164, 169, 171, 173, 175, 176, 179, 180, 183, 185, 191, 193, 195, 198 and 200;

(b) complements of the sequences recited in SEQ ID NOS: 142, 144, 146, 151, 153, 155, 157, 159, 161, 163, 164, 169, 171, 173, 175, 176, 179, 180, 183, 185, 191, 193, 195, 198 and 200; and

(c) sequences having at least about a 99% probability of being the same as a sequence of (a) or (b) as measured by computer algorithm BLASTN.

4. An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide according to any one of claims 1-3.

5. An expression vector comprising a polynucleotide according to claim 4.

6. A host cell transformed with an expression vector according to claim 5.

7. The host cell of claim 6, wherein the host cell is selected from the group consisting of *E. coli*, mycobacteria, insect, yeast and mammalian cells.

8. A fusion protein comprising at least one polypeptide according to any one of claims 1-3.

9. A pharmaceutical composition comprising a polypeptide according to any one of claims 1-3 and a physiologically acceptable carrier.

10. A pharmaceutical composition comprising a polynucleotide according to claim 4 and a physiologically acceptable carrier.

11. A pharmaceutical composition comprising a fusion protein according to claim 8 and a physiologically acceptable carrier.

12. A vaccine comprising a polypeptide according to any one of claims 1-3 and a non-specific immune response amplifier.

13. A vaccine comprising a polynucleotide according to claim 4 and a non-specific immune response amplifier.

14. A vaccine comprising a fusion protein according to claim 8 and a non-specific immune response amplifier.

15. A vaccine according to any one of claims 12-14 wherein the non-specific immune response amplifier is an adjuvant.

16. A vaccine according to any one of claims 12-14 wherein the non-specific immune response amplifier is selected from the group consisting of:

(a) delipidated and deglycolipidated *M. vaccae* cells;

(b) inactivated *M. vaccae* cells; and

(c) *M. vaccae* culture filtrate.

17. A method for enhancing an immune response in a patient, comprising administering to a patient a pharmaceutical composition according to any one of claims 9-11.

18. A method for enhancing an immune response in a patient, comprising administering to a patient a vaccine according to any one of claims 12-14.

19. The method of any one of claims 17 and 18, wherein the immune response is a Th1 response.

20. A method for the treatment of a disorder in a patient, comprising administering to the patient a pharmaceutical composition according to any one of claims 9-11.

21. A method for the treatment of a disorder in a patient, comprising administering to the patient a vaccine according to any one of claims 12-14.

22. The method of any one of claims 20 and 21, wherein the disorder is selected from the group consisting of immune disorders, infectious diseases, skin diseases and diseases of the respiratory system.

23. The method of claim 23 wherein the disorder is selected from the group consisting of mycobacterial infections, asthma, and psoriasis.

24. A method for the treatment of a disorder in a patient comprising administering a composition comprising a component selected from the group consisting of:

(a) inactivated *M. vaccae* cells;

(b) delipidated and deglycolipidated *M. vaccae* cells;

(c) delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids;

(d) delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids and arabinogalactan; and

(e) *M. vaccae* culture filtrate,

the disorder being selected from the group consisting of immune disorders, infectious diseases, skin diseases and diseases of the respiratory system.

25. The method of claim 24, wherein the disorder is selected from the group consisting of mycobacterial infections, asthma and psoriasis.

26. A method for enhancing a non-specific immune response to an antigen comprising administering a polypeptide, the polypeptide comprising an immunogenic portion of a *M. vaccae* antigen, wherein the *M. vaccae* antigen includes a sequence selected from the group consisting of:

(a) sequences recited in SEQ ID NO: 89 and 201; and

(b) sequences having at least about 80% identical residues to a sequence recited in SEQ ID NO: 89 and 201 as determined by computer algorithm BLASTP.

27. A method for detecting mycobacterial infection in a patient, comprising:

(a) contacting dermal cells of a patient with one or more polypeptides according to any one of claims 1-3; and

(b) detecting an immune response on the patient's skin.

28. The method of claim 27 wherein the immune response is induration.

29. A diagnostic kit comprising:

(a) a polypeptide according to any one of claims 1-3; and

(b) apparatus sufficient to contact the polypeptide with the dermal cells of a patient.

30. A method for detecting mycobacterial infection in a biological sample, comprising:

(a) contacting the biological sample with a polypeptide according to any one of claims 1-3; and

(b) detecting in the sample the presence of antibodies that bind to the polypeptide.

31. The method of claim 30 wherein the polypeptide(s) are bound to a solid support.

32. The method of claim 30 wherein the biological sample is selected from the group consisting of whole blood, serum, plasma, saliva, cerebrospinal fluid and urine.

33. A method for detecting mycobacterial infection in a biological sample, comprising:

(a) contacting the biological sample with a binding agent which is capable of binding to a polypeptide according to any one of claims 1-3; and

(b) detecting in the sample a protein or polypeptide that binds to the binding agent.

34. The method of claim 33 wherein the binding agent is a monoclonal antibody.

35. The method of claim 33 wherein the binding agent is a polyclonal antibody.

36. A diagnostic kit comprising:

(a) at least one polypeptide according to any one of claims 1-3; and

(b) a detection reagent.

37. The kit of claim 36 wherein the polypeptide is immobilized on a solid support.

38. The kit of claim 36 wherein the detection reagent comprises a reporter group conjugated to a binding agent.

39. The kit of claim 38 wherein the binding agent is selected from the group consisting of anti-immunoglobulins, Protein G, Protein A and lectins.

40. The kit of claim 38 wherein the reporter group is selected from the group consisting of radioisotopes, fluorescent groups, luminescent groups, enzymes, biotin and dye particles.

41. A monoclonal antibody that binds to a polypeptide according to any one of claims 1-3.

42. A polyclonal antibody that binds to a polypeptide according to any one of claims 1-3.

43. A method for enhancing a non-specific immune response to an antigen comprising administering a composition comprising a component selected from the group consisting of:

(a) delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids; and

(b) delipidated and deglycolipidated *M.vaccae* cells depleted of mycolic acids and arabinogalactan.