Vaccines Displaying Mycobacterial Proteins on Biopolyester Beads Stimulate Cellular Immunity and Induce Protection against Tuberculosis

Natalie A. Parlane,a,b Katrin Grage,b Jun Mifune,b Randall J. Basaraba,c D. Neil Wedlock,a Bernd H. A. Rehm,b and Bryce M. Buddlea
AgResearch, Hopkirk Research Institute, Palmerston North, New Zealand; Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand; and Colorado State University, Department of Microbiology, Immunology and Pathology, Fort Collins, Colorado, USA.

New improved vaccines are needed for control of both bovine and human tuberculosis. Tuberculosis protein vaccines have advantages with regard to safety and ease of manufacture, but efficacy against tuberculosis has been difficult to achieve. Protective cellular immune responses can be preferentially induced when antigens are displayed on small particles. In this study, Escherichia coli and Lactococcus lactis were engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which displayed a fusion protein of Mycobacterium tuberculosis, antigen 85A (Ag85A)—early secreted antigenic target 6-kDa protein (ESAT-6). L. lactis was chosen as a possible production host due its extensive use in the food industry and reduced risk of lipopolysaccharide contamination. Mice were vaccinated with PHB bead vaccines with or without displaying Ag85A–ESAT-6, recombinant Ag85A–ESAT-6, or M. bovis BCG. Separate groups of mice were used to measure immune responses and assess protection against an aerosol M. bovis challenge. Increased amounts of antigen-specific gamma interferon, interleukin-17A (IL-17A), IL-6, and tumor necrosis factor alpha were produced from splenocytes postvaccination, but no or minimal IL-4, IL-5, or IL-10 was produced, indicating Th1- and Th17-biased T cell responses. Decreased lung bacterial counts and less extensive foci of inflammation were observed in lungs of mice receiving BCG or PHB bead vaccines displaying Ag85A–ESAT-6 produced in either E. coli or L. lactis compared to those observed in the lungs of phosphate-buffered saline-treated control mice. No differences between those receiving wild-type PHB beads and those receiving recombinant Ag85A–ESAT-6 were observed. This versatile particulate vaccine delivery system incorporates a relatively simple production process using safe bacteria, and the results show that it is an effective delivery system for a tuberculosis protein vaccine.

Mycobacterium bovis, the causative agent of bovine tuberculosis (TB), infects a wide range of hosts, including domestic livestock and wildlife, and also causes TB in humans. Bovine TB poses a public health risk, particularly in regions where pasteurization of milk is not routine. This is of particular concern because more than 94% of the world’s population lives in such regions, and M. bovis is the causative agent for up to 10% of TB cases in humans in these regions (14). Bovine TB also has a considerable economic impact on the agricultural industry. The human TB vaccine Mycobacterium bovis bacille Calmette-Guérin (BCG) is only partially effective in both cattle and humans (2, 12). Development of an effective vaccine protecting against bovine TB would provide a cost-effective TB control strategy as well as have applicability for control of human TB caused by Mycobacterium tuberculosis.

A number of new TB vaccines are entering human clinical trials, including recombinant BCG, virus-vectored vaccines, and recombinant protein vaccines (20). One of the major constraints in developing effective recombinant protein vaccines is the difficulty of inducing the strong cellular immune responses which are required for protection against this disease. Selection of appropriate adjuvants and presentation of the proteins are critical. A number of studies have shown that antigens displayed on small particles preferentially enhance cellular immune responses to antigens (32, 47). Particles used to display antigens in vaccines include virus-like particles, liposomes, immune-stimulating complexes, and biological polymers, and these have been tested in a wide range of veterinary and wildlife species (45). The particles appear to have adjuvancing effects, with uptake by dendritic cells and consequen-

Received 29 August 2011 Returned for modification 11 October 2011 Accepted 25 October 2011

1556-6811/12/$12.00 Clinical and Vaccine Immunology p. 37–44 cvi.asm.org 37

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/CVI.05505-11
cells can be utilized to advantage for vaccine development (26). Similarly, Ag85A has long been recognized to be an immunodominant antigen (22) and is incorporated in a recombinant modified vaccinia virus Ankara-expressing Ag85A vaccine currently in human trials as a BCG boosting vaccine (27, 35). The Ag85 complex consists of three proteins which possess mycolyl transferase activity and play a role in the biogenesis of the mycobacterial cell wall (7).

A disadvantage of using E. coli as a production host for products with in vivo use is the contamination with lipopolysaccharide (LPS) endotoxins. However, processes used to remove LPS are costly and can destroy surface proteins and the functionality of the particles (55). *Lactococcus lactis*, an organism which does not produce LPS, has been used extensively in the food industry and has recently been used for recombinant protein production and delivery of therapeutic agents, vaccines, and TB skin-test reagents (8, 30, 52, 56). Therefore, *L. lactis* might be a suitable production host for PHB bead TB vaccines.

This paper describes engineering of the food-grade bacterium *L. lactis* to produce biopolyester PHB beads displaying mycobacterial antigens Ag85A and ESAT-6. Vaccines were prepared from PHB beads produced in *L. lactis* or *E. coli* and used to immunize mice. Immune responses were measured, and following aerosol challenge with *M. bovis*, the protective immunity against TB was assessed.

### MATERIALS AND METHODS

**Construction of plasmids for production of PHB beads displaying Ag85A–ESAT-6.** Plasmids used in this study are listed in the supplemental material. General cloning procedures and DNA isolation were carried out as described elsewhere (44). Biosynthesis of PHB polyester requires genes for enzymes PhaA, PhaB, and PhaC. To construct pNZ-Ag85E6-CAB for use in *L. lactis*, the gene encoding fusion of the antigens Ag85A and ESAT-6 was synthesized by GeneScript Corporation (Piscataway, NJ). Codon usage was adapted to the codon usage bias of *E. coli*. A fragment of pUC57-ZZ comprising part of the nisA promoter (PnisA) was obtained by NdeI digest of pUC57-ZZ and ligated with NdeI-digested pUC57-Ag85E6 to obtain pUC57-nisAg85E6. A BstBI-BamHI fragment of pUC57-nisAg85E6 containing the section of PnisA and the Ag85A–ESAT-6 gene was then inserted upstream of phaB at the corresponding sites of pNZ-AB, resulting in pNZ-Ag85E6-B. To introduce the phaC- and phaA-comprising fragment of pNZ-CAB into pNZ-Ag85E6-B, both plasmids were hydrolyzed with NheI and BamHI and the phaCA fragment of pNZ-CAB was inserted into pNZ-Ag85E6-B, resulting in pNZ-Ag85E6-CAB. This plasmid was electroporated into *L. lactis* (NZ9000) for subsequent production of PHB beads. For production of wild-type control PHB beads, the genes (phaA, phaB, and phaC) were engineered into *E. coli* BL21 (DE3) and *L. lactis* NZ2900 using methods previously described (28, 39), and construction of plasmids for production of Ag85A–ESAT-6 PHB beads in *E. coli* was undertaken as described elsewhere (37).

**Bacterial strains, growth conditions, and isolation of PHB beads.**

*E. coli* strains were grown in Luria broth (LB; Difco, Detroit, MI) supplemented with 1% (wt/vol) glucose, ampicillin (75 µg/ml), and chloramphenicol (30 µg/ml). *L. lactis* strains were grown in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% glucose, 0.3% 1-arginine, and chloramphenicol (10 µg/ml).

PHB beads which displayed Ag85A–ESAT-6 or control PHB beads alone were produced in *E. coli* and *L. lactis* as previously described (28, 37). Briefly, *E. coli* was grown at 30°C in LB, induced with 1 mM isopropyl β-D-thiogalactopyranoside to produce protein, and cultured for a further 48 h to allow accumulation of beads. *L. lactis* cultures were produced in M17 broth, induced with 10 mg/ml nisin to produce protein, and cultured for a further 48 h at 30°C. Presence of PHB/polyester was determined by staining the cultures with Nile red lipophilic dye and then using fluorescence microscopy to observe fluorescence associated with the intracellular beads (38). Bacteria were then mechanically disrupted, and *E. coli* lysate was centrifuged at 4,000 × g and *L. lactis* lysate was centrifuged at 8,000 × g for 15 min at 4°C to sediment the polyester beads. All beads were then purified via glycerol gradient ultracentrifugation. Polyester production was determined by measuring the PHB content of the granules using gas chromatography-mass spectroscopy (GC-MS) (10).

**Analysis of proteins attached to PHA beads.** The concentration of proteins attached to the PHB beads was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose using an i-BLOT system (Invitrogen). A mouse monoclonal primary antibody against ESAT-6 (Abcam, Cambridge, United Kingdom) was used at a 1:800 dilution. Following incubation with rabbit anti-mouse peroxidase-conjugated immunoglobulin G (Dako, Carpinteria, CA), development was carried out using aminomethyl carbazole. To confirm the identity of the protein of interest, a band was excised from the gel and subjected to tryptic peptide fingerprinting using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). Specific activity of the Ag85A–ESAT-6 PHB beads was determined by enzyme-linked immunosorbent assay (ELISA) as previously described (37).

**Recombinant protein antigen and peptides.** Recombinant Ag85A–ESAT-6 protein (rec Ag85A–ESAT-6) was produced as previously described (48) with some variation. Briefly, *E. coli* BL21 Star (DE3)pLysS (Invitrogen) was transformed with pAg85-ESAT-6 in pET32A (a kind gift from Lynne Slobbe, Otago University, New Zealand) and grown in Terrific broth. The insoluble recombinant protein in the cell culture pellet was solubilized in 6 M urea, and proteins were separated by SDS-PAGE using NuPAGE gels. The band of interest was excised from the gel and protein was eluted using a Bio-Rad model 422 electrophoresis (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. The protein was then refolded by dialysis in decreasing concentrations of urea, desalted using a desalt column (Pierce, Rockford, IL), and treated with polymyxin B-agarose (Sigma Chemicals, St. Louis, MO) to remove contaminating LPS. The activity of the rec Ag85A–ESAT-6 was then confirmed by ELISA.

**Vaccination of mice.** Vaccines comprising PHB beads displaying Ag85A–ESAT-6 antigen produced in *E. coli* (EcAgE) and *L. lactis* (LcAgE) were adjusted to contain 30 µg of Ag85A–ESAT-6–PhaC protein, as calculated from the densitometry profile. Similarly, control wild-type (WT) vaccines produced in *E. coli* (EcWT) and *L. lactis* (LcWT) were adjusted to contain 30 µg of the PhaC protein alone. Emulsigen (MVP Laboratories, Omaha, NE) adjuvant (20%, vol/vol) was mixed with the various PHB beads, 30 µg rec Ag85A–ESAT-6, or phosphate-buffered saline (PBS).

Female C57BL/6 mice aged 6 to 8 weeks (purchased from the animal breeding facility of the Malaghan Institute of Medical Research, Wellington, New Zealand) were vaccinated 3 times subcutaneously with 200 µl injection at 9-day intervals (n = 12 per group). A control group received a single dose of 10° CFU of BCG Pasteur strain 1173P2 (a kind gift from the Malaghan Institute of Medical Research). All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

**Cell preparations and immunological assays.** Five weeks after the first vaccination, half of the animals in each group were euthanized, spleens were removed, and a single-cell suspension was prepared by passage through an 80-gauge wire mesh sieve. Spleen red blood cells were lysed using a solution of 17 mM Tris-HCl and 140 mM NH₄Cl. After washing, the cells were cultured in Dulbecco’s modified Eagle medium.
(DMEM; Invitrogen) supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma), 1× nonessential amino acids (Gibco, Grand Island, NY), and 5% (wt/vol) fetal bovine serum (Invitrogen) in triplicate wells of flat-bottomed 96-well plates at a concentration of 5 × 10⁵ cells/well in a 200-μl volume. The cells were incubated with medium alone or in medium containing a pool of Ag85A, ESAT-6, or Ag85A and ESAT-6 overlapping peptides (final concentration, 5 μg/ml; Auspep, Victoria, Australia). Concanavalin A (5 μg/ml; Sigma) was used as a positive control. Cells were incubated at 37°C in an atmosphere of 10% CO₂ in air. Spleen cells from BCG-vaccinated mice were also cultured with bovine purified protein derivative (PPD; 5 μg/ml; Prionics AG, Switzerland).

**Measurement of cytokines in culture supernatants.** Levels of IFN-γ and interleukin-5 (IL-5) in culture supernatants were measured by ELISA using commercial pairs of antibodies and standards (BD, Franklin Lakes, NJ). The assay used o-phenylenediamine substrate and was read at 495 nm on a VERSAmax microplate reader. Standard curves were constructed using SOFTmax PRO software, and the averages of cytokine values of duplicate samples were determined from the curve. A cytometric bead array (CBA; mouse Th1-Th2 cytokine kit; BD) was used according to the manufacturer’s instructions to measure other cytokines: IL-2, IL-4, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), and IL-17A. Fluorescence was measured using a FACScalibur flow cytometer (BD) and analyzed using FCAP array software (BD). Results for all cytokines were calculated as the cytokine value of the Ag85A–ESAT-6-stimulated sample minus that of the PBS-stimulated sample.

**Mycobacterium bovis challenge and necropsy.** Fifteen weeks after the first vaccination, all remaining mice (n = 6 per group) were challenged with M. bovis (strain 83/6235) by the aerosol route. M. bovis was grown from a low-passage seed lot in Tween albumin broth (Tween 80, Dubos broth base, and oleic acid-albumin-dextrose [Difco, BD Diagnostic Systems, Sparks, MD]) to early mid-log phase, and aliquots of cultures were frozen at −70°C until required. To infect mice by low-dose aerosol exposure, diluted thawed stock was administered using a Madison chamber aerosol generation device calibrated to deliver approximately 50 bacteria into the lungs. Aerosol infections, maintenance, and manipulation of infected mice were performed under strict isolation conditions in a biohazard facility.

Five weeks after challenge with M. bovis, the mice were euthanized and spleens and lungs were removed. The right apical lung lobe was removed from the lung and preserved in 10% buffered formalin, for subsequent histological processing, followed by staining of sections with Ziehl-Neelsen stain and hematoxylin and eosin (H&E) stain. The lung lesion areas were quantified relative to total lung area on randomly selected, H&E-stained tissue sections. The total lung and lesion areas were quantified using a stereology-based method referred to as the area fractionator with the investigator blinded to the treatment groups. The area of inflammation relative to total lung area was estimated from sections evaluated at ×200 magnification. A total of 8 to 12 fields was randomly selected by the computer, and a counting frame (2,000 μm²) containing probe points with a grid spacing of 200 μm was used to define the areas of interest. The data are expressed as the mean percentage of lung affected by lesions of all the animals within a treatment group (n = 6). Lesions represented by photomicrographs are for individuals that have a value closest to the mean value for the entire treatment group.

**Mycobacterial culture of lungs and spleens.** Spleen and remaining lung samples were mechanically homogenized in 3 ml of PBS with 0.5% Tween 80 using a Seward Stomacher 80 device (Seward, Norfolk, United Kingdom) and plated in 10-fold dilutions on selective Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco). Plates were incubated at 37°C in humidified air for 3 weeks before colonies were counted.

**Statistical analysis.** Cytokine responses were analyzed using the Kruskal-Wallis test. Data for analyses of histopathology percent lung lesion involvement were square root transformed and bacterial counts from the M. bovis-challenged mice were log₁₀ transformed, and the transformed data were compared by Fisher’s one-way analysis of variance. The level of significance was set at a P value of <0.05.

**RESULTS**

Production and characterization of biopolyester beads displaying Ag85A–ESAT-6 in L. lactis and E. coli. Plasmids encoding polyester synthase and Ag85A–ESAT-6 were successfully introduced into L. lactis, which enabled production of beads displaying Ag85A–ESAT-6. The presence of intracellular inclusions was observed by fluorescence microscopy using Nile red staining (see the supplemental material), and GC-MS analysis of cells confirmed the presence of the polyester, PHB (data not shown). E. coli was also used to produce beads displaying Ag85A–ESAT-6 as previously described (37). The sizes of the beads were shown to be 50 to 150 nm for L. lactis-produced beads and 150 to 250 nm for beads produced in E. coli. Following purification of the beads from L. lactis hosts, the proteins associated with the Ag85A–ESAT-6–PhaC beads and the wild-type PhaC control beads were separated by SDS-PAGE. Proteins with molecular masses similar to the molecular masses of 102 kDa for the Ag85A–ESAT-6–PhaC fusion and 63 kDa for PhaC were observed, and Western blot analysis with anti-ESAT-6 antibody demonstrated a predominant band at approximately 102 kDa which corresponds to the Ag85A–ESAT-6–PhaC fusion (Fig. 1). The identity of the Ag85A–ESAT-6–PhaC band was confirmed by tryptic peptide fingerprinting using MALDI-TOF MS (see the supplemental material). PHB beads displaying Ag85A–ESAT-6 from E. coli and L. lactis hosts were shown...

**FIG 1** (A) SDS-PAGE analysis of proteins attached to polyester beads. PHB beads were isolated from L. lactis NZ9000 harboring the following plasmids: pNZ-CAB (lane 1) and pNZ-Ag85E6-CAB (lane 2). Lane M, molecular mass markers. (B) Western blot: reactivity of proteins to ESAT-6 antibody. Lane 1, PHB beads isolated from L. lactis NZ9000 harboring plasmid pNZ-Ag85E6-CAB; lane 2, recombinant ESAT-6; lane M, molecular mass markers.
by ELISA to bind to anti-ESAT-6 antibody in a dose-dependent manner (see the supplemental material).

**Clinical and immunological responses to vaccination.** Mouse weights did not differ significantly between groups during the time course of the experiment, and mice in all groups steadily gained weight. Mice vaccinated with PHB beads developed small lumps up to 2.5 mm in diameter at the vaccination sites with no signs of abscess or suppuration. No lumps were observed in the other vaccine groups.

To assess development of Th1 cell-mediated immunity, splenocytes were restimulated in vitro with a pool of Ag85A and ESAT-6 peptides, and released cytokines were measured. Vaccination of mice with PHB beads displaying Ag85A–ESAT-6 produced in both *E. coli* and *L. lactis* stimulated the generation of an antigen-specific cellular immune response compared to the PBS-vaccinated group. The vaccine groups receiving PHB beads displaying Ag85A–ESAT-6 produced in both *E. coli* and *L. lactis* produced significantly more IFN-γ, IL-2, IL-6, TNF-α, and IL-17A than the group receiving PBS (Fig. 2) (*P* < 0.05). The only significant increase in cytokines released from splenocytes of the rec Ag85A–ESAT-6 group compared to PBS control mice was for IL-2 (Fig. 2B) (*P* < 0.05). The wild-type control PHB groups did not show any significant increase in any of these cytokines measured compared to PBS-vaccinated mice. IL-10 responses were very low, and there were no significant differences between groups (Fig. 2). IL-5 and IL-4 were measured but were detected only in very small amounts and only in one to two animals in any group (data not shown). The results of IFN-γ release from splenocytes stimulated with Ag85A or ESAT-6 peptides or the combined pool of Ag85A and ESAT-6 peptides are shown in the supplemental material. Responses for the groups vaccinated with PHB beads displaying Ag85A–ESAT-6 produced in both *E. coli* and *L. lactis* were strongest for the pool of Ag85A and ESAT-6 peptides and weakest for the Ag85A peptides. BCG-vaccinated mice produced a significant increase in release of IFN-γ from splenocytes stimulated with bovine PPD compared to the PBS-vaccinated group (data not shown).

**Histopathology.** The *M. bovis*-infected lung lobes from the PBS-vaccinated mice had multiple, coalescing foci of granulomatous inflammation composed predominantly of epithelioid macrophages and lymphocytes (Fig. 3). In acid-fast (AF) stained sections, intracellular bacilli were observed in many of the
macrophages that made up the lesions (data not shown). The lungs of mice vaccinated with BCG or PHB beads displaying Ag85A–ESAT-6 produced in E. coli or L. lactis had loosely organized accumulations of inflammatory cells. These lesions were smaller, were less extensive with fewer lymphocytes and macrophages than those from PBS-vaccinated mice, and were often within the perivascular parenchyma. The lung lesions in mice vaccinated with wild-type PHB beads or rec Ag85A–ESAT-6 were similar to those in the PBS controls. Morphometric analysis was used to determine the percentage of normal parenchyma replaced by inflammatory lesions. The lung lesions in mice vaccinated with wild-type PHB beads or rec Ag85A–ESAT-6 were significantly less involved than those vaccinated with wild-type PHB beads produced in either E. coli or L. lactis or the PBS-vaccinated group (Fig. 4) (P < 0.05). PHB beads displaying Ag85A–ESAT-6 produced in E. coli had significantly less lung lesion involvement than wild-type beads produced in E. coli (P < 0.05). Although PHB beads displaying Ag85A–ESAT-6 produced in L. lactis had less lung lesion involvement than wild-type beads produced in L. lactis, this difference was not statistically significant (P = 0.11). Differences between the various groups vaccinated with the PHB beads and the PBS control group were not significant.

Mycobacterial culture. A significant reduction in the bacterial counts was observed from the lungs of animals receiving Ag85A–ESAT-6 PHB bead vaccines produced in either E. coli or L. lactis or the BCG vaccine compared to the PBS-vaccinated negative-control group (Fig. 5A) (P < 0.05). M. bovis culture results of spleens showed that animals vaccinated with Ag85A–ESAT-6 PHB bead vaccines produced in E. coli and BCG had a significant reduction in spleen bacterial counts compared to the PBS-vaccinated group (Fig. 5B) (P < 0.05). There were no significant
differences between the mean lung counts for animals receiving Ag85A–ESAT-6 PHB bead vaccines and animals in the BCG group, while the mean spleen counts for the Ag85A–ESAT-6 PHB bead vaccine groups were significantly higher than the mean for the BCG group (P < 0.05). The group vaccinated with Ag85A–ESAT-6 PHB beads produced in E. coli also had significantly lower mean lung and spleen counts than those in the rec Ag85A–ESAT-6 group (P < 0.05), while differences between the group vaccinated with Ag85A–ESAT-6 PHB beads produced in L. lactis and the rec Ag85A–ESAT-6 group were not significant. No significant reduction in bacterial counts from the lungs or spleens was observed from animals vaccinated with wild-type PHB beads or rec Ag85A–ESAT-6.

**DISCUSSION**

The novel vaccine delivery system based on biopolymer beads produced in E. coli has previously been shown to stimulate an immune response to mycobacterial antigens (37). This latest study demonstrated that the generally regarded as safe (GRAS) bacterium L. lactis could be used to produce vaccine beads displaying Ag85A–ESAT-6, and following vaccination with these beads, immune responses were similar to those obtained using beads from an E. coli production host. Furthermore, when mice vaccinated with Ag85A–ESAT-6 PHB beads produced in either E. coli or L. lactis were challenged with M. bovis, the significant reduction in lung bacterial counts was similar to that achieved with the “gold standard” BCG vaccine. The presence of LPS endotoxin from E. coli–produced recombinant proteins and vaccines limits the use of these products in humans without costly and potentially protein-destructive depyrogenation processes. Major advantages of using an L. lactis production host are the safety record and reduced risk of LPS endotoxin contamination. As well as its long history of safe use in the food industry, L. lactis is now being used for a range of other in vivo and in vitro applications (5, 6) and has recently been used to produce a vaccine antigen for a human clinical malaria vaccine trial (16). The safety of the PHB polyester used in these vaccines has been recognized, with FDA approval for PHB sutures (42).

Cytokine responses in the present study were indicative of a predominantly cell-mediated immune response when animals were vaccinated with PHB beads displaying Ag85A–ESAT-6 compared to animals vaccinated with PBS or rec Ag85A–ESAT-6 antigen alone in Emulsigen adjuvant. The antigen-specific increase in IFN-γ and TNF-α with minimal IL-4, IL-5, and IL-10 responses suggested that Th1-type immunity rather than Th2-type immunity was induced. Increases in IL-17A indicated that Th17 immunity was also stimulated. The IFN-γ response to the pool of peptides from Ag85A and ESAT-6 was greater than that to the peptides from the individual proteins. This suggested that the effect of vaccinating with the subunit vaccine and BCG could be additive compared to vaccinating with BCG alone. While it is generally accepted that IFN-γ has a significant role in protection from TB, there is no single postvaccination correlate of protection. Recent studies have shown that IFN-γ does not correlate with BCG-induced protection (29, 49), and others have determined that an increase in polyfunctional T cells, which produce IL-2, IFN-γ, and TNF-α, is thought to be an important possible correlate of protection (1). IL-17A (21), along with IL-6 and TNF-α (17, 24), appears to have a role in vaccination-induced immunity against TB, which concurs with the increased levels of these cytokines measured in the current study. Therefore, in the absence of absolute correlates of protection, challenge studies are necessary to determine vaccine efficacy.

The increased cytokine responses correlated with decreased bacterial counts and reduced pathology observed in the lungs. Histopathology results showed a distinct difference in granulomas in mice which had been vaccinated with BCG or PHB beads displaying Ag85A–ESAT-6, with these mice having smaller, less extensive foci of inflammation and fewer lymphocytes in the granulomas than PBS- or PHB wild-type bead-vaccinated animals. The significant reduction in spleen bacterial counts from BCG- and E. coli-produced Ag85A–ESAT-6 PHB bead-vaccinated animals indicates that extrapulmonary spread of tuberculosis has been minimized. It is unclear why L. lactis-produced Ag85A–ESAT-6 PHB bead-vaccinated animals did not instigate a similar reduction. The current PHB bead formulation may prove effective as a vaccine boost following BCG priming. Cytokine responses, culture results, and pathology demonstrated that mice vaccinated with recombinant Ag85A–ESAT-6 were unable to mount a protective response from M. bovis challenge. These results suggested that particulate vaccines were more effective than vaccines containing soluble antigens, a finding demonstrated in other vaccine studies (23, 43). The recombinant Ag85A–ESAT-6 was shown to be immunogenic, as this vaccine induced an IgG response to the mycobacterial peptides (data not shown).

While these novel vaccines have shown efficacy against TB, there are modifications which could be made to further enhance
their usefulness. PHB beads which display cytokine proteins have previously been produced (4), and both the N terminus and C terminus have been used to produce fusions for functional protein display (3). Therefore, it is likely that PHB beads which display both vaccine antigens and immunomodulator proteins on one bead could be produced, allowing codeelivery of vaccine agents to dendritic cells, which has been shown to increase immune responsiveness (9, 51). Alternatively, immunomodulators could be incorporated on the PHB beads by chemical conjugation. It is also likely that increased antigen could be displayed on beads by incorporating multiple gene repeats in the bacterial production strains (31). Another strategy which utilized both N-terminus and C-terminus fusions of PhAC (19) could be applied to enable production of two different vaccine antigens on the one bead and therefore produce multivalent vaccines. Combinations of PHB bead vaccines and BCG could result in the induction of enhanced protection against TB. Studies in cattle have shown that concurrent administration of TB protein vaccines and BCG produced better protection against bovine TB than administration of BCG alone (53, 54), and a BCG prime and TB protein boost is being advocated for use in humans (2).

The vaccine production process described here allows modification of the host genome so that alternative genes for vaccine antigens could be used. Most vaccines use immunodominant antigens, but more recent views (13, 33, 34, 50) suggest that use of subdominant antigens might be more appropriate for diseases for which there is no effective vaccine. PHB bead vaccines could also be developed and used for other vaccines where stimulation of cell-mediated immunity is required. Alternative antigen display has recently been demonstrated on PHB beads produced in E. coli and L. lactis. These beads displayed hepatitis C core antigen and were able to produce antigen-specific immune responses following vaccination (36). In conclusion, the current results indicate that vaccines based on PHB beads provide a platform for display of a range of antigens coupled with relatively simple production processes in safe bacteria.

ACKNOWLEDGMENTS

Many thanks go to Raj Palanisamy for preparing the initial batch of beads produced in L. lactis. Also, thanks go to Geoff DeLisle for supply of the M. bovis challenge strain, Dongwen Luo for statistical analyses, and the Grasslands animal facility staff for care and welfare of mice.

Natalie A. Parlane was supported in part by an AGMARDT Ph.D. scholarship. The research was funded by a New Zealand Foundation for Research, Science and Technology grant.

REFERENCES

1. Abel B, et al. 2010. The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. Am. J. Respir. Crit. Care Med. 181:1407–1417.
2. Andersen P, Doherty TM. 2005. The success and failure of BCG—implications for a novel tuberculosis vaccine. Nat. Rev. Microbiol. 3:656–662.
3. Atwood JA, Rehm BHA. 2009. Protein engineering towards biotechnological production of bifunctional polyester beads. Biotechnol. Lett. 31:131–137.
4. Bickelström BT, Brockelbank JA, Rehm BHA. 2007. Recombinant Escherichia coli produces tailor-made biopolymer granules for applications in fluorescence activated cell sorting: functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein. BMC Biotechnol. 17:3.
5. Bahey-El-Din M, Gahan CGM. 2011. Lactococcus lactis-based vaccines: current status and future perspectives. Hum. Vaccin. 7:106–109.
6. Bahey-El-Din M, Gahan CGM, Griffin BT. 2010. Lactococcus lactis as a cell factory for delivery of therapeutic proteins. Curr. Gene Ther. 10:34–45.
7. Belisle JT, et al. 1997. Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis. Science 276:1420–1422.
8. Bergstedt W, et al. 2010. First-in-man open clinical trial of a combined mTB7-6 and rCEFP-10 tuberculosis specific skin test reagent. PLoS One 5:e11277.
9. Borges O, et al. 2008. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. Int. Immunopharmacol. 8:1773–1780.
10. Brandl H, Gross RA, Lenz RW, Fuller RC. 1988. Pseudomonas oleovorans as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54:1977–1982.
11. Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P. 2000. ESAT-6 subunit vaccination against Mycobacterium tuberculosis. Infect. Immun. 68:791–795.
12. Budde BM. 2010. Tuberculosis vaccines for cattle: the way forward. Expert Rev. Vaccines 9:1121–1124.
13. Comas I, et al. 2010. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nat. Genet. 42:498–503.
14. Cosivi O, et al. 1998. Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. Emerg. Infect. Dis. 4:59–70.
15. Deng YH, Sun Z, Yang XL, Bao L. 2010. Improved immunogenicity of recombinant Mycobacterium bovis bacillus Calmette-Guérin strains expressing fusion protein Ag85A-ESAT-6 of Mycobacterium tuberculosis. Press YH, Sun Z, Yang XL, Bao L. 2010. Improved immunogenicity of recombinant Mycobacterium bovis bacillus Calmette-Guérin strains expressing fusion protein Ag85A-ESAT-6 of Mycobacterium tuberculosis. Scand. J. Immunol. 72:332–338.
16. Esen M, et al. 2009. Safety and immunogenicity of GMZ2—a MSP2-GLURP fusion protein malaria vaccine candidate. Vaccine 27:6862–6868.
17. Freches D, et al. 2011. Increased pulmonary tumor necrosis factor alpha, interleukin-6 (IL-6), and IL-17A responses compensate for decreased gamma interferon production in anti-IL-12 vaccine-treated, Mycobacterium bovis BCG-vaccinated mice. Clin. Vaccine Immunol. 18:95–104.
18. Harboe M, Oettinger T, Wiiker HG, Rosenkrands I, Andersen P. 1996. Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and its absence in Mycobacterium bovis BCG. Infect. Immun. 64:16–22.
19. Jahns AC, Rehm BHA. 2009. Tolerance of the Ratibiosis estropho class I polyhydroxyalkanoate synthase for translational fusions to its C terminus reveals a new mode of functional display. Appl. Environ. Microbiol. 75:5461–5466.
20. Kaufmann SHE, Hussey G, Lambert P-H. 2010. New vaccines for tuberculosis. Lancet 375:2110–2119.
21. Khader SA, et al. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat. Immunol. 8:369–377.
22. Launois P, et al. 1994. T-cell epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. Infect. Immun. 62:3675–3678.
23. Liang MT, Davies NM, Blanchfield JT, Torh I. 2006. Particulate systems as adjuvants and carriers for peptide and protein antigens. Curr. Drug Deliv. 3:379–388.
24. Lin Y, Slight S, Khader S. 2010. Th17 cytokines and vaccine-induced immunity. Semin. Immunopathol. 32:79–90.
25. Lu J, et al. 2011. Immunogenicity and protective efficacy against murine tuberculosis of a prime-boost regimen with BCG and a DNA vaccine expressing ESAT-6 and Ag85A fusion protein. Clin. Dev. Immunol. 2011:617892.
26. Majlessi I, et al. 2005. Influence of ESAT-6 secretion system 1 (RDI1) of Mycobacterium tuberculosis on the interaction between mycobacteria and the host immune system. J. Immunol. 174:3570–3579.
27. McShane H, et al. 2004. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat. Med. 10:1240–1244.
28. Mifune J, Grage K, Rehm BHA. 2009. Production of functionalized biopolymer granules by recombinant Lactococcus lactis. Appl. Environ. Microbiol. 75:4668–4675.
29. Mittrucker HW, et al. 2007. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc. Natl. Acad. Sci. U. S. A. 104:12434–12439.
30. Morello E, et al. 2008. Lactococcus lactis, an efficient cell factory for
recombinant protein production and secretion. J. Mol. Microbiol. Biotechnol. 14:48–58.

31. Mullaney JA, Rehm BHA. 2010. Design of a single-chain multi-enzyme fusion protein establishing the polyhydroxybutyrate biosynthesis pathway. J. Biotechnol. 147:31–36.

32. Newman KD, Samuel J, Kwon G. 1998. Ovalbumin peptide encapsulated in poly[(R)-lactic-co-glycolic acid] microspheres is capable of inducing a T helper type 1 immune response. J. Control. Release 54:49–59.

33. Ordway D, et al. 2007. The hypervirulent Mycobacterium tuberculosis strain HN878 induces a potent TH1 response followed by rapid down-regulation. J. Immunol. 179:522–531.

34. Orme IM. 2011. Development of new vaccines and drugs for TB: Limitations and potential strategic errors. Future Microbiol. 6:161–177.

35. Parida SK, Kaufmann SHE. 2010. Novel tuberculosis vaccines on the horizon. Curr. Opin. Immunol. 22:374–384.

36. Parlane NA, et al. 7 October 2011, posting date. Production of a particulate hepatitis C vaccine candidate by engineered Lactococcus lactis. Appl. Environ. Microbiol. doi:10.1128/AEM.06420-11.

37. Parlane NA, Wedlock DN, Buddle BM, Rehm BHA. 2009. Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agent. Appl. Environ. Microbiol. 75:7739–7744.

38. Peters V, Becher D, Rehm BH. 2007. The inherent property of polyhydroxalkanoate synthase to form spherical PHA granules at the cell poles: the core region is required for polar localization. J. Biotecnol. 132:238–245.

39. Peters V, Rehm BHA. 2008. Protein engineering of streptavidin for in vivo assembly of streptavidin beads. J. Biotechnol. 134:266–274.

40. Pollock JM, Andersen P. 1997. Predominant recognition of the ESAT-6 protein in the first phase of infection with Mycobacterium bovis in cattle. Infect. Immun. 65:2587–2592.

41. Ravn P, et al. 1999. Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. J. Infect. Dis. 179:637–645.

42. Rebell H. 2007. FDA clears first of its kind suture made using DNA technology. P07-18.FDA, Washington, DC.

43. Rice-Ficht AC, Arenas-Gamboa AM, Kahl-McDonagh MM, Ficht TA. 2010. Polymeric particles in vaccine delivery. Curr. Opin. Microbiol. 13:106–112.

44. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a labora-

45. Scheerlinck JP, Greenwood DL. 2006. Particulate delivery systems for animal vaccines. Methods 40:118–124.

46. Sharp FA, et al. 2009. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc. Natl. Acad. Sci. U. S. A. 106:870–875.

47. Singh M, Chakrapani A, O’Hagan D. 2007. Nanoparticles and microparticles as vaccine-delivery systems. Expert Rev. Vaccines 6:797–808.

48. Skinner MA, et al. 2003. A DNA prime-live vaccine boost strategy in mice can augment IFN-gamma responses to mycobacterial antigens but does not increase the protective efficacy of two attenuated strains of Mycobacterium bovis against bovine tuberculosis. Immunology 108:548–555.

49. Soares AP, et al. 2008. Bacillus Calmette-Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. J. Immunol. 180:3569–3577.

50. Tobin GJ, et al. 2008. Deceptive imprinting and immune refocusing in vaccine design. Vaccine 26:6189–6199.

51. Tricas JA, Sun I, Palendira U, Britton WJ. 2002. Comparative affects of plasmid-encoded interleukin 12 and interleukin 18 on the protective efficacy of DNA vaccination against Mycobacterium tuberculosis. Immunol. Cell Biol. 80:346–350.

52. Villatoro-Hernandez J, Montes-De-Oca-Luna R, Kuipers OP. 2011. Targeting diseases with genetically engineered Lactococcus lactis and its course towards medical translation. Expert Opin. Biol. Ther. 11:261–267.

53. Wedlock DN, et al. 2008. Enhanced protection against bovine tuberculosis after coadministration of Mycobacterium bovis BCG with a mycobacterial protein vaccine-adjuvant combination but not after coadministration of adjuvant alone. Clin. Vaccine Immunol. 15:765–772.

54. Wedlock DN, et al. 2005. Vaccination of cattle with a CpG oligodeoxynucleotide-formulated mycobacterial protein vaccine and Mycobacterium bovis BCG induces levels of protection against bovine tuberculosis superior to those induced by vaccination with BCG alone. Infect. Immum. 73:3540–3546.

55. Williams SF, Martin DP, Horowitz DM, Peoples OP. 1999. PHA applications: addressing the price performance issue. I. Tissue engineering. Int. J. Biol. Macromol. 25:111–121.

56. Zhang Q, Zhong J, Huan L. 2011. Expression of hepatitis B virus surface antigen determinants in Lactococcus lactis for oral vaccination. Microbiol. Res. 166:111–120.