Evaluation of the Mtb72F Polyprotein Vaccine in a Rabbit Model of Tuberculous Meningitis

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Received 12 September 2005/Returned for modification 9 November 2005/Accepted 26 January 2006

Using a rabbit model of tuberculous meningitis, we evaluated the protective efficacy of vaccination with the recombinant polyprotein Mtb72F, which is formulated in two alternative adjuvants, AS02A and AS01B, and compared this to vaccination with Mycobacterium bovis bacillus Calmette-Guérin (BCG) alone or as a BCG prime/Mtb72F-boost regimen. Vaccination with Mtb72F formulated in AS02A (Mtb72F+AS02A) or Mtb72F formulated in AS01B (Mtb72F+AS01B) was protective against central nervous system (CNS) challenge with Mycobacterium tuberculosis H37Rv to an extent comparable to that of vaccination with BCG. Similar accelerated clearances of bacilli from the cerebrospinal fluid, reduced leukocytosis, and less pathology of the brain and lungs were noted. Weight loss of infected rabbits was less extensive for Mtb72F+AS01B-vaccinated rabbits. In addition, protection against M. tuberculosis H37Rv CNS infection afforded by BCG/Mtb72F in a prime-boost strategy was similar to that by BCG alone. Interestingly, Mtb72F+AS01B induced better protection against leukocytosis and weight loss, suggesting that the polyprotein in this adjuvant may boost immunity without exacerbating inflammation in previously BCG-vaccinated individuals.

The development of improved tuberculosis (TB) vaccines has been recognized as a major public health priority by the World Health Organization. At present, the attenuated strain of Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only vaccine that is widely used against TB (7, 17). The BCG vaccine is relatively effective against the progression of infection to disseminated TB and tuberculous meningoencephalitis (TBM) in children (38). It has limited efficacy, however, against pulmonary TB in adults (16, 35, 48). An improved vaccine that protects both children against TB and adults against pulmonary TB disease would be very useful in combating TB. Consequently, a number of research groups are engaged in developing more effective anti-TB vaccines by utilizing either new attenuated live vaccines (4, 12, 20, 22, 39, 40, 47, 49) or subunit protein vaccines (3, 6, 11, 26, 34, 37, 41). While attenuated live vaccines provide prolonged exposure of the host immune system to newly synthesized antigens, the advantage of subunit vaccines is the possibility that their efficacy may not be compromised by exposure to environmental mycobacteria (5) or by prior BCG vaccination (8).

To adequately predict the efficacy of any new anti-TB vaccine in humans, it is imperative to develop assays for measuring the level of protection that the vaccines provide. Animal models of infection are useful tools for this purpose. In our previous studies, we established and characterized a rabbit model of TBM which mirrors human central nervous system (CNS) disease (45). In this model, mycobacteria are inoculated directly into the cisterna magna of rabbits and the course of infection and the host response are monitored. We are using this animal model of CNS infection to evaluate the efficacy of new candidate vaccines for protecting the host against both infection and the pathology of TBM.

Recently, several Mycobacterium tuberculosis antigens were characterized for their ability to elicit T-cell and antibody responses (1, 13, 25, 43). Among these, two proteins, Mtb32 and Mtb39, were expressed as a recombinant fusion polyprotein designated Mtb72F (13, 42). The immunization of mice with Mtb72F DNA or recombinant protein formulated in two different adjuvant systems, AS01B and AS02A (GlaxoSmithKline Biologicals proprietary adjuvants), resulted in the induction of strong immune responses to the components of Mtb72F. In addition, vaccinated mice were protected against aerosol challenge with a virulent strain of M. tuberculosis (41). Similarly, the immunization of guinea pigs with Mtb72F delivered either as DNA or as recombinant antigen in adjuvant prolonged the survival of the animals following aerosol challenge with virulent mycobacteria (41). Here we evaluated the protective efficacy of Mtb72F formulated in AS01B (Mtb72F+AS01B) or in AS02A (Mtb72F+AS02A) against disease caused by M. tuberculosis infection in the CNS of rabbits. In addition, because newborns are vaccinated with BCG in most countries in which TB is endemic, we also evaluated whether Mtb72F+AS01B or Mtb72F+AS02A can improve the protective efficacy of BCG in a prime-boost strategy. We monitored CNS bacillary load, dissemination of the bacilli across the blood-brain barrier to other organs, weight loss (an important clinical sign of disease), and tissue pathology and compared the efficacy of the new candidate vaccine to that of BCG alone.
MATERIALS AND METHODS

Vaccination of animals. Outbred New Zealand White rabbits (weight, approximately 2.0 kg; Covance Research Products, Inc., Denver, PA) were used for this study. Rabbits were vaccinated three times (at 3-week intervals) intramuscularly with 20 μg of Mtb72F+AS02A or Mtb72F+AS01B (GlaxoSmithKline Biologicals, Rixensart, Belgium) in a total volume of 500 μl (2). Another group of rabbits was first primed subcutaneously with 0.1 ml BCG (5 × 10⁶ CFU) (Danish 1331;SSI, Copenhagen, Denmark) and 6 weeks later were boosted 3 times with Mtb72F+AS02A or Mtb72F+AS01B. Some rabbits received only the BCG vaccine. All animals were challenged 10 weeks after the last immunization by an intrathecal injection of 5 × 10⁶ CFU M. tuberculosis H37Rv (TMC no. 102, Trudeau Institute, Saranac Lake, NY). One group of infected but not vaccinated animals served as a negative control.

IgG isotype ELISA. Four rabbits that were vaccinated with Mtb72F+AS02A were used for blood collection 3 weeks after the last immunization. Two BCG-vaccinated and three nonvaccinated rabbits were used for comparisons. Plasma was stored at -70°C. The specific immunoglobulin G (IgG) isotype antibody response to the Mtb72 polyprotein was measured by conventional enzyme-linked immunosorbent assay (ELISA) as previously described (41).

Lymphocyte proliferation assay. Ficoll-separated (Amersham Pharmacia Biotech AB, Uppsala, Sweden) rabbit peripheral blood mononuclear cells were stimulated with BCG (Danish 1331) at a multiplicity of infection of 0.2 for 6 days in supplemented 20% fetal calf serum (GemCell; Gemini BioProducts, Woodland, CA)-RPML (Invitrogen, Carlsbad, CA). On day 6, cells were pulsed with bromodeoxyuridine (BrdU) at a concentration of 10 μM (BrdU flow kit, BD Pharmingen, San Jose, CA) for 5 h and then harvested and processed according to the kit protocol. Cells were stained with mouse anti-rabbit CD4-fluorescein isothiocyanate (Spring Valley Laboratories, Woodbine, MD) and anti-BrdU (BD Pharmingen) antibodies. The percentage of CD4+ cells that incorporated BrdU was assessed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

Induction of meningitis. Ten weeks after the third immunization with Mtb72F+AS02A or Mtb72F+AS01B, a helmet of dental acrylic was preattached to the rabbits' skulls to facilitate immobilization in a stereotaxic frame as previously described (45). During the next day, animals were anesthetized and immobilized; a spinal needle was used to withdraw 0.3 ml of cerebrospinal fluid (CSF) and inject 0.2 ml of 5 × 10⁶ CFU of M. tuberculosis H37Rv intracisternally. Clinical and immunologic parameters were monitored until 5 weeks postinfection, when the rabbits were euthanized. At this time, half of the brains and segments of lung and liver were collected aseptically, homogenized, and used for CFU determinations. The remaining brain, lung, and liver tissues were fixed in 10% buffered formalin acetate (vol/vol) (Fisher Chemical, Fairlawn, NJ) for histology. The protocol for these experiments was approved by the IACUC at UMDNJ—Newark campus and PHRI, Newark, NJ.

CSF samples. CSF samples were analyzed for leukocyte counts (Coulter Electronics, Inc., Hialeah, FL), and 0.1 ml was used for the CFU assay. The remaining CSF was centrifuged at 10,000 × g for 5 min, and the supernatant was stored at -70°C for the tumor necrosis factor (TNF) assay.

Blood samples. Heparinized blood was collected from the ear artery and centrifuged at 10,000 × g. Plasma was separated and frozen at -70°C for the TNF evaluation.

TNF assay. Since there is no commercially available ELISA kit for rabbits, TNF biologic activity was evaluated by using a cytotoxicity assay in murine L929 fibroblasts as described previously (45). This assay does not distinguish between TNF-α and TNF-β; thus, we refer to the cytokine as TNF.

CFU assay. The CFU were evaluated in the CSF and organ homogenates by plating 10-fold serial dilutions onto Middlebrook 7H11 agar (Difco), as described previously (45).

Histopathology. Formalin-fixed brains were cut transversely in serial sections from rostral to caudal, representing the fore-, mid-, and hindbrains. Tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin and Ziehl-Neelsen for microscopic evaluation.

To evaluate pathological changes in the CNS and lungs of infected and vaccinated rabbits, we developed scoring systems for brains and meninges (0, normal; 1, mild meningitis; 2, moderate focal meningitis; 3, moderate meningitis with vasculitis; and 4, severe meningitis and encephalitis) and lungs (0, normal parenchyma; 1, increased cellularity; 2, one to two small organized granulomas per section [approximately 1 square cm]; 3, more than two larger lesions per section with central necrosis; and 4, large confluent lesions with acid-fast bacilli). All histologic sections were evaluated blind by a single investigator.

Statistical analysis. The Mann-Whitney test for nonparametric independent data or the one-way analyses of variance was used for these studies. A P value of <0.05 was considered significant.

RESULTS

The rabbit antibody response to the Mtb72F polyprotein. To ascertain that the Mtb72F polyprotein is specifically recognized by the rabbit immune system, animals were immunized with Mtb72F+AS02A and evaluated for plasma anti-Mtb72F IgG antibody titers 3 weeks after the final vaccination. All four rabbits developed strong IgG responses against the antigen (Fig. 1). No Mtb72F-specific responses were detected in BCG-vaccinated or control nonvaccinated rabbits.

Effect of AS01B and AS02A adjuvant on the Mtb72F-induced protection against CNS M. tuberculosis infection and inflammation. We compared the protective efficacy of vaccination with Mtb72F+AS01B, Mtb72F+AS02A, and BCG in groups of four rabbits. At 2 h postinfection, approximately 4 log₁₀ CFU/ml CSF were detected in all animals from all groups (not shown). In nonvaccinated control rabbits, the bacillary load dropped to 1 to 2 log₁₀ CFU/ml CSF by 1 week postinfection, remained relatively high until week 4, and then slowly dropped but never cleared (Fig. 2A). In contrast, by week 1, CSF bacillary loads for rabbits that were immunized with BCG, Mtb72F+AS01B, or Mtb72F+AS02A were significantly lower (P values were 0.0004, 0.008, and 0.0007, respectively) than those for control nonvaccinated animals (Fig. 2A). By week 3 to 4 postinfection, no viable bacilli were detected in the CSF of any rabbits in the three vaccination groups. No statistically significant differences were observed among the animals from the vaccination groups. At 8 weeks postinfection, animals were euthanized and bacillary counts in the brain were determined. Brain bacillary loads in rabbits vaccinated with BCG, Mtb72F+AS02A, or Mtb72F+AS01B were not significantly different from the controls, although there was a trend towards reduction in the two former groups (4.4 log₁₀ ± 0.4 for nonvaccinated, 3.7 log₁₀ ± 0.2 for BCG, 3.7 log₁₀ ± 0.3 for Mtb72F+AS02A, and 4.6 log₁₀ ± 0.1 for Mtb72F+AS01B).

A major criterion for meningeal inflammation is leukocyte influx into the CSF. M. tuberculosis infection of control nonvaccinated rabbits resulted in the early recruitment and persistence of leukocytes in the CSF (Fig. 2B). Differential counts revealed a polymorphonuclear leukocyte response in the CSF for the first...
Effect of AS01B and AS02A adjuvant on Mtb72F-induced protection against CNS M. tuberculosis infection and inflammation (n = 4 rabbits/group). Values are means. Error bars indicate standard errors of the means. (A) Number of CFU of M. tuberculosis in the CSF for control nonvaccinated rabbits (black bars), BCG-vaccinated rabbits (white bars), rabbits vaccinated with Mtb72F+AS01B (dark gray bars), rabbits vaccinated with Mtb72F+AS02A (light gray bars), and rabbits vaccinated with Mtb72F+AS01B+AS02A (gray bars). Bacillary clearance was significantly accelerated in all vaccinated groups compared to that of the control nonvaccinated animals (P = 0.0004 for BCG, 0.0007 for Mtb72F+AS02A, and 0.0008 for Mtb72F+AS01B). (B) WBC density in CSF for control nonvaccinated rabbits (black squares), rabbits vaccinated with BCG (open squares), rabbits vaccinated with Mtb72F+AS02A (open triangles), and rabbits vaccinated with Mtb72F+AS01B (×). Leukocyte counts were significantly reduced in all vaccinated groups compared to those of nonvaccinated rabbits (P = 0.0001 for BCG, 0.0002 for Mtb72F+AS02A, and 0.0003 for Mtb72F+AS01B), with no significant difference among the vaccinated groups. (C) Body weight change (%) for control nonvaccinated rabbits (black squares), rabbits vaccinated with BCG (open squares), rabbits vaccinated with Mtb72F+AS02A (open triangles), and rabbits vaccinated with Mtb72F+AS01B (×). Rabbits vaccinated with Mtb72F+AS02A showed the least change in the body weight compared to that of the control nonvaccinated animals (P = 0.0001).

FIG. 2. Effect of AS01B and AS02A adjuvant on Mtb72F-induced protection against CNS infection and inflammation (n = 4 rabbits/group). Values are means. Error bars indicate standard errors of the means. (A) Number of CFU of M. tuberculosis in the CSF for control nonvaccinated rabbits (black bars), BCG-vaccinated rabbits (white bars), rabbits vaccinated with Mtb72F+AS01B (dark gray bars), rabbits vaccinated with Mtb72F+AS02A (light gray bars), and rabbits vaccinated with Mtb72F+AS01B+AS02A (gray bars). Bacillary clearance was significantly accelerated in all vaccinated groups compared to that of the control nonvaccinated animals (P = 0.0004 for BCG, 0.0007 for Mtb72F+AS02A, and 0.0008 for Mtb72F+AS01B). (B) WBC density in CSF for control nonvaccinated rabbits (black squares), rabbits vaccinated with BCG (open squares), rabbits vaccinated with Mtb72F+AS02A (open triangles), and rabbits vaccinated with Mtb72F+AS01B (×). Leukocyte counts were significantly reduced in all vaccinated groups compared to those of nonvaccinated rabbits (P = 0.0001 for BCG, 0.0002 for Mtb72F+AS02A, and 0.0003 for Mtb72F+AS01B), with no significant difference among the vaccinated groups. (C) Body weight change (%) for control nonvaccinated rabbits (black squares), rabbits vaccinated with BCG (open squares), rabbits vaccinated with Mtb72F+AS02A (open triangles), and rabbits vaccinated with Mtb72F+AS01B (×). Rabbits vaccinated with Mtb72F+AS02A showed the least change in the body weight compared to that of the control nonvaccinated animals (P = 0.0001).

24 h after infection, followed by a shift to mononuclear cells (>95%). Throughout the experiment, leukocyte counts were significantly lower in all vaccinated groups in comparison to nonvaccinated rabbits (P values were < 0.0001 for BCG, 0.0002 for Mtb72F+AS02A, and 0.0003 for Mtb72F+AS01B). Weight loss, a manifestation of TB disease, was more extensive in nonvaccinated control animals relative to all vaccination groups. Protection from weight loss was most prominent in the Mtb72F+AS02A vaccinated animals (P = 0.0001); this result was better even than that for BCG (P = 0.0001) (Fig. 2C).

Taken together, these results suggest that a subunit vaccine with adjuvants (AS02A or AS01B) can induce a level of protection against TBM similar to that conferred by BCG. Since Mtb72F+AS02A showed better protection against weight loss, this formulation was used in the next set of experiments.

Effect of Mtb72F+AS02A or BCG vaccination of rabbits on bacillary load in the CNS and in other tissues. Eight rabbits per group were vaccinated, challenged, and evaluated as described above. The clearance of bacilli from the CSF was already improved by 1 week postinfecion and sustained in both vaccinated groups relative to the nonvaccinated control animals (P was <0.0001 for BCG and Mtb72F+AS02A) (Fig. 3A). By 4 weeks, a low bacillary load was detected in controls, while no viable organisms were detected in the CSF of rabbits that were vaccinated with Mtb72F+AS02A or BCG. In addition, at 8 weeks postinfection, significantly smaller amounts of CFU were observed in the brains of rabbits vaccinated with BCG (P = 0.03) or Mtb72F+AS02A (P = 0.02) compared to those observed with controls (Fig. 3B). The dissemination of bacilli to the lungs was significantly reduced in BCG-vaccinated rabbits (P = 0.03) and, to a lesser extent (not significant), in animals that were immunized with Mtb72F+AS02A. While the liver was infected in all nonvaccinated control animals, no liver infection was noted in any animals from the two vaccinated groups (P = 0.002) (Fig. 3B). These results confirm and expand our pilot experiment, showing that vaccination with Mtb72F+AS02A accelerated the clearance of bacilli in a manner similar to that of BCG.

Effect of vaccination with Mtb72F+AS02A on the inflammatory response, body weight, and pathology in brain and lungs of infected rabbits. CSF leukocytosis in infected nonvaccinated animals peaked at 2 weeks (Fig. 4A). This early level of leukocytosis in BCG- or Mtb72F+AS02A-vaccinated animals was significantly lower throughout the experiment (P was 0.0004 and <0.0001, respectively). Elevated bacillary loads and increased CSF leukocytosis in the CNS are usually associated with local TNF-α production. In this study, the TNF levels in the CSF were very low to undetectable in all rabbits (not shown).

Nonvaccinated rabbits progressively lost more than 15% of their body weight over the 8 weeks of infection. In contrast, BCG-vaccinated animals maintained their weight for the first 4 weeks (Fig. 4B) and then lost 5% of their body weight (P was 0.02 relative to controls). Vaccination with Mtb72F+AS02A resulted in better protection from weight loss than did BCG (P = 0.03).

Control nonvaccinated rabbits developed moderate meningitis, with large numbers of inflammatory cells in the subarachnoid space, and partial vasculitis (brain pathology score of 2.75 from a maximum of 4) (Fig. 4C). All vaccinated rabbits showed only mild-to-moderate focal inflammation of the meninges, with no signs of vasculitis in the BCG-vaccinated animals. Pathology in the CNSs of BCG-vaccinated rabbits (score, 1.4)
and, to a lesser extent, of animals vaccinated with Mtb72F/H11001 AS02A (score, 1.9) was significantly attenuated in comparison to that of controls. No significant differences were noted between the two vaccination groups. In spite of the presence of various levels of brain pathology, no neurologic signs, such as loss of coordination, paresis, and paralysis of the limbs, were noted in any of the rabbits, including the control nonvaccinated animals.

Increased cellularity in the parenchyma, thickening of the alveolar walls, and two to four large confluent granulomas per section, with central necrosis and single acid-fast bacilli, were observed in the lungs of control nonvaccinated rabbits (lung pathology score of 3.8 from a maximum of 4). Significantly attenuated pathology was observed in the lungs of rabbits from both vaccination groups (scores of 0.8 for BCG and 1.5 for Mtb72F in AS02A) (P was <0.001 and 0.008, respectively) (Fig. 4C). None or very few well-organized small granulomas and no acid-fast bacilli were observed in the lungs of vaccinated ani-

(P = 0.01) and, to a lesser extent, of animals vaccinated with Mtb72F+AS02A (score, 1.9) was significantly attenuated in comparison to that of controls. No significant differences were noted between the two vaccination groups. In spite of the presence of various levels of brain pathology, no neurologic signs, such as loss of coordination, paresis, and paralysis of the limbs, were noted in any of the rabbits, including the control nonvaccinated animals.

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mals. No significant differences were noted between the two vaccinated groups of animals. Thus, the vaccination of rabbits with Mtb72F+AS02A or with BCG resulted in reductions in inflammation and pathology of brain and lungs similar to those of nonvaccinated rabbits following infection with M. tuberculosis.

**Effect of boosting with Mtb72F+AS02A or Mtb72F+AS01B on bacillary numbers and pathology in the CNS and lungs of BCG-vaccinated rabbits.** We compared the efficacy of vaccination with BCG alone to that of vaccination with BCG prime followed by a boost with Mtb72F+AS02A or Mtb72F+AS01B, in four animals per group. The clearance of CSF CFU was significantly accelerated in the three vaccination groups compared to that in nonvaccinated controls (P=0.01 for BCG, 0.02 for BCG followed by Mtb72F+AS02A, and 0.01 for BCG followed by Mtb72F+AS01B) (Table 1). Vaccination was also associated with reduced bacillary loads in the brain and lungs of rabbits from all vaccination groups. However, this effect was statistically significant, relative to controls, in the brains of only animals that were vaccinated with BCG and boosted with Mtb72F+AS01B (P<0.05). Similarly, a reduction in leukocytosis (white blood cells [WBC]) was observed in all groups of vaccinated rabbits. However, the BCG prime/Mtb72F+AS01B-boost animals were the only group that showed a statistically significant difference relative to nonvaccinated controls (P=0.02). BCG alone and BCG boosted with Mtb72F+AS01B induced significant reductions in weight loss compared to that induced by the control animals (P=0.03 and 0.01, respectively) (Table 1).

Histologic examination of the brains of control rabbits infected with M. tuberculosis revealed moderate inflammation of the meninges (distended subarachnoid space, high numbers of mononuclear cells, and partial necrosis (Fig. 5A and Table 1). Vasculitis was observed not only in the meninges but also in the brain parenchyma (neuropil) (Fig. 5a). In contrast, much milder focal inflammation of the meninges, predominantly in the sulcus area, with no signs of vasculitis in the brain, was observed in the BCG-vaccinated animals (Fig. 5B and Table 1). Rabbits vaccinated with Mtb72F+AS02A showed some vasculitis in the meninges (Fig. 5C) and none in the brain parenchyma (Fig. 5c). No significant differences in the alleviation of tissue pathology were noted between the two adjuvant systems (Table 1). However, animals that were vaccinated with BCG and boosted with Mtb72F+AS01B developed very mild or no meningeal inflammation (Fig. 5D), with no signs of vasculitis (P=0.006) (Fig. 5d).

While control rabbits developed large confluent lung granulomas with foamy nondifferentiated macrophages, lymphocytes, and areas of necrosis (Fig. 6A and a; Table 1), only a few small granulomas were observed in the lungs of animals that were vaccinated with Mtb72F+AS02A (Fig. 6C and c). In these granulomas, macrophages appeared epithelioid (activated) and surrounded by large numbers of lymphocytes. No granulomas were found in the lungs of the BCG-vaccinated rabbits (Fig. 6B and b) or rabbits that were vaccinated with BCG and boosted with Mtb72F+AS02A (Fig. 6D and d). Increased accumulation of mononuclear leukocytes in the alveolar walls was noted in some of the animals from both groups (Fig. 6b and d); no acid-fast bacilli were detected in their lungs. The semiquantitative analysis of the lung histology revealed a statistically significant reduction in lung pathology of animals that were BCG vaccinated and those that were BCG vaccinated and boosted with Mtb72F+AS02A (P=0.001).

To test whether the vaccine-induced protection was associated with the induction of an acquired T-cell response, rabbits that were immunized with BCG alone or vaccinated with BCG and boosted with Mtb72F+AS01B were evaluated for BCG-induced CD4+ T-cell proliferation, which was evaluated by BrdU incorporation measured by flow cytometry. BCG vaccination of rabbits induced a statistically significant (P=0.02) increase in antigen-specific T-cell proliferation compared to that of nonvaccinated controls (Fig. 7). Animals that were vaccinated with BCG and boosted with Mtb72F+AS01B demonstrated T-cell activation similar to that observed following vaccination with BCG only. Thus, the level of BCG-specific T-cell activation correlated with the extent of protection.

**DISCUSSION**

We have demonstrated that the vaccination of rabbits with Mtb72F, formulated in an AS02A or AS01B adjuvant, is as protective as BCG against CNS challenge with M. tuberculosis. Similarly to vaccination with BCG, the accelerated clearance of bacilli from the CSF reduced leukocytosis, and attenuated...
FIG. 5. Histopathology of the meninges and brain of rabbits infected intrathecally with *M. tuberculosis* H37Rv (8 weeks postinfection). (A and a) Control nonvaccinated rabbit. (A) Moderate inflammation of the meninges with high numbers of inflammatory cells, lymphocytes, and macrophages (mac) and partial necrosis (nec). (a) Vasculitis is seen not only in the meninges but also in the brain parenchyma. (B and b) Mild focal inflammation within the meninges with no evidence of vasculitis for rabbit vaccinated with BCG. (C and c) Moderate meningitis associated with perivascular inflammation for a rabbit vaccinated with Mtb72F+AS02A. Note the lymphocytes infiltrating the vessel wall (C). No signs of vasculitis were observed in the brain parenchyma (c). (D and d) Rabbit vaccinated with BCG and boosted with Mtb72F+AS01B. Very mild focal inflammation is seen in the meninges with few lymphocytes within the subarachnoid space (arrows), and no signs of vasculitis are seen. Blood vessels are labeled V. Sections are stained with hematoxylin and eosin. Magnification, ×40.
CD8 robust and comprehensive immune response (CD4 Mtb72F formulated in AS01B was shown to induce a more also been noted previously in the mouse infection model (41). The differences in the rabbit response to vaccination with the two adjuvants that were observed in our study have (44, 46). The differences in the rabbit response to vaccination compared to that observed with Mtb72F formulated in AS02A (41). In our experiments in naïve rabbits, Mtb72F+AS01B was less protective; in previously BCG-vaccinated rabbits, Mtb72F+AS01B showed better protection. Thus, prior immunity appeared to influence the relative efficacy of the two adjuvants. This may suggest that the contribution of adjuvant-induced innate immunity to protection versus inflammation and pathology may be a function of the immune status of the animal. Thus, in humans too, prior immune activation may determine whether the administration of any given new vaccine results in better protection or in increased inflammation and pathology. This possibility needs to be further explored.

Our observations confirm and expand the reported efficacy of Mtb72F in M. tuberculosis-infected mice and guinea pigs (41). Furthermore, given that the rabbits were challenged intrathecally at 10 weeks after the third immunization, the protective efficacy against CNS infection, which was induced by Mtb72F in adjuvant, appears to be relatively long lasting. One advantage of using a polyprotein vaccine rather than BCG in humans is that viable BCG can cause a difficult-to-treat opportunistic infection in human immunodeficiency virus-infected neonates as well as adults (9, 15, 29).

Currently, the mouse and guinea pig aerosol tuberculosis infection models are the most widely used for TB vaccine evaluation. Some authors consider mice more resistant to TB than are humans (36). The inflammatory response in mice is less pronounced, and they do not develop typical lung pathology. Nevertheless, the mouse model is useful for studying the host immune response to TB. Guinea pigs are more susceptible than humans, but develop lung granulomas with necrosis similar to those in humans (31). While our rabbit model of TBM may not fully reflect the natural history of CNS infection in humans, this model resembles human TBM clinically and histopathologically and facilitates studying disease and pathogenesis in the CNS as well as other organs (45). Previous studies by others (10, 27) have also shown that pulmonary tuberculosis in rabbits can be an excellent model of human tuberculosis, since both species develop caseous lesions and cavities. Clearly, the rabbit model has some limitations, including the paucity of commercial immunologic reagents. In addition, inbred rabbits are not available; consequently, experiments are done in outbred animals, giving rise to larger variations in results within each vaccination group. Nevertheless, this diversity would also be the case when any vaccine is tested in humans.

BCG, the only available and widely used tuberculosis vaccine, protects children against miliary TB and TBM (16). However, it fails to protect children against other milder forms of childhood TB or adults against pulmonary TB. Thus, a new TB vaccine that protects adults is urgently needed (21, 35). Because of the protective efficacy of BCG in early childhood, the BCG vaccination of newborn children, especially in regions where TB is prevalent, should be continued. Consequently, new vaccines will be tested and potentially administered in previously BCG-vaccinated individuals. This need is addressed by the prime-boost approach that we have tested (8, 32, 33). In our experiments, the boosting of BCG with Mtb72F+AS01B resulted in a significant reduction in the inflammatory response (P = 0.02 for WBC in CSF), less weight loss as well as a reduced bacillary load, and attenuated pathology in the brains of infected rabbits (Table 1). Thus, it appears that the protective efficacy of BCG prime-boost is at least as good as that of BCG alone. The efficacy of Mtb72F-DNA as a boost in already BCG-vaccinated guinea pigs has also been recently reported (6). Animals boosted with Mtb72F-DNA showed better resolution and earlier healing of lung lesions.
One of the current views in TB vaccine design is that existing memory immunity should be targeted with a specific antigen that is recognized by a sufficient number of memory CD4⁺ T cells (8). Recently, a few prime-boost studies, using RV3407 DNA (33), Ag85A (8), or MVA85A (modified vaccinia virus Ankara expressing antigen 85A) (32) as a boost, appeared to achieve superior protection compared to that conferred by BCG vaccination alone in mice and improved immunity in humans.

In conclusion, our results provide evidence that the recombinant polypeptide Mtb72F, formulated either in AS01B or AS02A, is efficacious in the rabbit model of TB and represents a valuable candidate vaccine for testing in countries in which TB is endemic. Moreover, the boosting of animals that are vaccinated with BCG with Mtb72F in adjuvant does not interfere with the protective efficacy against M. tuberculosis challenge, suggesting that future clinical trials can be done on previously BCG-vaccinated individuals.

ACKNOWLEDGMENTS

Funding for these studies was provided in part by NIH grant AI054338 (to G.K.) and by Corixa Corporation.

We thank Yves Lobet for scientific assistance and Ulrike Krause and Dorothy Fallows for critically reviewing the manuscript and valuable suggestions.

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Editor: J. L. Flynn