Tuberculosis (TB) remains a major health problem affecting millions of people worldwide. The only TB vaccine presently available is an attenuated strain of *Mycobacterium bovis* termed *M. bovis* BCG. The efficacy of BCG remains controversial, particularly against pulmonary TB in young adults (5), and development of a better vaccine is urgently needed to counter the global threat of this disease (22).

Secreted and surface-exposed cell wall proteins are major antigens recognized by the protective immune response against TB and immunization with whole-culture filtrate, a rich source of these extracellular proteins, can protect mice and guinea pigs to some extent against subsequent challenge with the tubercle bacillus (1, 14, 15). A major portion of the secreted proteins in *Mycobacterium tuberculosis* and BCG culture filtrate is formed by the Ag85 complex, a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C) (38) which all possess a mycoloyltransferase enzyme activity required for the biogenesis of cord factor (4), a dominant structure necessary for maintaining cell wall integrity (19, 29). Ag85 complex induces strong T-cell proliferation and gamma interferon (IFN-γ) production in most healthy individuals infected with *M. tuberculosis* and/or *Mycobacterium leprae* (24) and in BCG-vaccinated mice (16), making it a promising candidate as a protective antigen. Vaccination with naked plasmid DNA encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated immune responses and confer significant protection to C57BL/6 (B6) mice challenged by the aerosol or intravenous route with live *M. tuberculosis* H37Rv (17, 20). Only intramuscular (i.m.) needle injection but not epidermal gene gun bombardment is capable of inducing a protective, Th1-biased immune response with this vaccine (36). In experimental mouse models, Ag85A DNA vaccine so far is effective only during the first weeks after *M. tuberculosis* challenge, and subsequently its protection, as measured by reduced CFU counts in lungs, wanes (37).

Here we report on an attempt to improve the immunogenicity and protective efficacy of this Ag85 DNA TB vaccine by a DNA prime-protein boost immunization regimen. Indeed, i.m. DNA vaccination is particularly effective in priming a Th1-type immune response, but the low amount of actual protein antigen synthesized in the host is a serious limitation of this type of immunization. Prime-boost strategies of consecutive DNA priming followed by boosting with purified proteins or with attenuated poxviruses have the potential to improve dramatically these DNA-based vaccines through preferential amplification of CD4+ or CD8+ effectors, respectively (27, 30). Whereas a number of studies have reported on the effect of protein boosting of DNA vaccines encoding viral (3, 25, 26, 28, 31, 35) and protozoal (12, 21) antigens, little is known with respect to mycobacterial infections. Here we demonstrate that protein boosting of B6 mice vaccinated with plasmid DNA encoding Ag85A and Ag85B from *M. tuberculosis* is capable of
increasing the immunogenicity and (to a lesser extent) protective efficacy of this experimental TB DNA vaccine.

MATERIALS AND METHODS

Plasmid construction. Plasmid DNAs encoding a mature or secreted form of Ag85A and Ag85B from M. tuberculosis were prepared as described previously (2).

**Mice.** B6 mice were bred in the Animal Facilities of the Pasteur Institute of Brussels. Only female mice 6 to 8 weeks old at the start of vaccination were used.

**Protein, DNA, and BCG vaccination.** For protein immunization, mice were injected intraperitoneally (i.p.) in the back with 100 μg of Ag85A purified by sequential chromatography from BCG culture filtrate (7) and emulsified in monophosphoryl lipid A (mpl-A) from Salmonella enterica serovar Minnesota (Ribi ImmunoChem Research, Hamilton, Mont.) solubilized in triethanolamine. The amino acid sequences of Ag85A from M. tuberculosis and of BCG are 100% identical (8). For DNA vaccination, mice were anesthetized by intraperitoneal injection of ketamine and xylazine (100 and 10 mg/kg of body weight, respectively) and injected i.m. in both quadriceps with 2 × 50 μg of plasmid DNA either in saline (Ag85A DNA) or complexed in the cationic lipid vaxfectin (Ag85B DNA) (13). For the DNA prime-protein boost, mice were immunized i.m. with Ag85 DNA and s.c. with 1, 10, 30, 50, or 100 μg of purified native Ag85A protein in mpl-A or with 50 μg of purified recombinant Ag85B protein (11) in SBASA22A adjuvant (SmithKline Beecham). All mice received three immunizations at 3-week intervals. For BCG vaccination, mice were injected intravenously (i.v.) in a lateral tail vein with 10^6 CFU of freshly prepared BCG (strain GL2) grown as a surface pellicle on synthetic Sauton medium (16) on the same day as the third immunization.

**ELISA.** Sera from immunized mice were collected by retro-orbital bleeding 2 months after the third vaccination. Levels of total anti-Ag85A IgG antibodies (Abs) were determined by enzyme-linked immunosorbent assay (ELISA) in sera from individual mice (five/group). The serum titer was converted to Ab concentration (nanograms per milliliter) by comparison with a standard monoclonal Ab, and mean Ab concentration was calculated from at least three points of the linear portion of the titration curve. Concentrations were converted to log_{10} values. For isotype analysis, peroxidase-labeled rat anti-mouse immunoglobulin G1 (IgG1) and IgG2a (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium) were used. Equal amounts of the five sera in each group were pooled, and isotype titers were determined and converted to arbitrary units by comparison with the titer of a standard serum pool from Ag85A DNA-immunized mice, arbitrarily assigned a titer of 1,000 for both isotypes.

**Cytokine production.** Vaccinated mice were sacrificed 3 weeks (dose-response experiment) or 2 months (peptide mapping) after the third immunization, and spleens were removed aseptically. Spleen cells from three mice per group were tested individually for cytokine response to whole Ag85A (5 μg/ml) or p25 (10 μg/ml) (dose-response experiment) and as a pool for peptide mapping (18). Supernatants were harvested after 24 h (interleukin-2 [IL-2]) and 72 h (IFN-γ), when peak values of the respective cytokines could be measured. Supernatants from at least three separate wells were pooled and stored frozen at −20°C until the assay. Analysis was performed twice, and data from one experiment are reported.

**IL-2 assay.** IL-2 activity was measured using a bioassay, as described previously (16). Each sample was tested in duplicate. IL-2 levels are expressed in mean cpm and SD values for three mice tested individually in spleen cell cultures stimulated for 24 h with purified Ag85A (5 μg/ml) 3 weeks after the last immunization.

**IFN-γ levels (picograms per milliliter [mean ± SD]) for three mice tested individually in spleen cell cultures stimulated for 72 h with p25 (amino acids 241 to 260, 10 μg/ml) 3 weeks after the last immunization.

**RESULTS**

Ag85A-specific IL-2 and IFN-γ production in spleen cell cultures from B6 mice vaccinated with Ag85A DNA and boosted with Ag85A protein

**TABLE 1. Ag85A-specific IL-2 and IFN-γ production in spleen cell cultures from B6 mice vaccinated with Ag85A DNA and boosted with Ag85A protein**

<table>
<thead>
<tr>
<th>Vaccine used (μg of protein)</th>
<th>IL-2 (cpm)a</th>
<th>IFN-γ (pg/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA 3×</td>
<td>16,393 ± 6,716</td>
<td>1,118 ± 915</td>
</tr>
<tr>
<td>DNA 2×-protein (1)</td>
<td>20,836 ± 8,414</td>
<td>787 ± 367</td>
</tr>
<tr>
<td>DNA 2×-protein (10)</td>
<td>14,501 ± 3,294</td>
<td>992 ± 374</td>
</tr>
<tr>
<td>DNA 2×-protein (30)</td>
<td>127,494 ± 57,549</td>
<td>5,521 ± 1,486</td>
</tr>
<tr>
<td>DNA 2×-protein (50)</td>
<td>84,841 ± 22,793</td>
<td>3,950 ± 1,327</td>
</tr>
</tbody>
</table>

a IL-2 levels (counts per minute [mean ± SD]) for three mice tested individually in spleen cell cultures stimulated for 24 h with purified Ag85A (5 μg/ml) 3 weeks after the last immunization.

b IFN-γ levels (picograms per milliliter [mean ± SD]) for three mice tested individually in spleen cell cultures stimulated for 72 h with p25 (amino acids 241 to 260, 10 μg/ml) 3 weeks after the last immunization.

**M. tuberculosis challenge.** B6 mice were rested for 2 months after the third immunization and were challenged i.v. in a lateral tail vein with 10^6 CFU of M. tuberculosis H37Rv (37) (Ag85A DNA) or with 10^5 CFU of recombinant luciferase reporter M. tuberculosis H37Rv (34) (Ag85B DNA). Mice vaccinated with Ag85A DNA were sacrificed 30, 60, or 90 days after challenge, and serial threefold total lung homogenate dilutions were plated on 7H11 Middlebrook agar supplemented with oleic acid-albumin-dextrose-catalase (OADC). Colonies were counted visually after 4 weeks. CFU counts obtained from two or three dilutions were used to calculate the total number of CFU/lung/mouse. For statistical analysis (Student’s t test), these data were converted to log_{10} values and log_{10} (mean ± SD) values for CFU/lung/mouse were calculated for each experimental group, which consisted of 3 to 10 animals tested individually (as indicated in Table 3). Mice vaccinated with Ag85B DNA were sacrificed 30 days after challenge, and the number of bacteria per lung was determined by classical CFU counting on Middlebrook 7H11 agar and in a bioluminescence assay using a Turner Design 20/20 luminometer and 1% n-decylaldehyde in ethanol as the substrate (34).
Ag85 specific IFN-γ production in CD4+ splenic T cells from DNA primed/protein boosted B6 mice

FIG. 1. Flow cytometry analysis of Ag85A-specific IFN-γ production on days 1 and 3 by CD4+ spleen T cells from B6 mice that were vaccinated with plasmid DNA encoding a mature form of Ag85A and boosted with increasing doses of purified Ag85A protein in MPL-A. FITC, fluorescein isothiocyanate.

Spleen cell IFN-γ production in B6 mice vaccinated with Ag85A protein, Ag85A DNA, or a DNA prime-protein boost regimen. Three vaccinations with 100 µg of purified Ag85A protein in MPL-A induced only a weak IFN-γ response to whole native Ag85A protein (768 ± 253 pg/ml) when animals were tested 2 months after the last immunization (Fig. 2). T-cell epitope mapping using synthetic 20-mer peptides spanning the entire mature Ag85A sequence from M. tuberculosis showed that following protein immunization, IFN-γ responses were the strongest against two peptide regions that have pre-

FIG. 2. Spleen cell IFN-γ response to whole Ag85A and its synthetic peptides in mice vaccinated with Ag85A protein or Ag85A DNA or in B6 mice vaccinated with Ag85A DNA and a protein boost 2 months after the third immunization.
Previously been identified as immunodominant in B6 mice vaccinated with live BCG (18) or infected with *M. tuberculosis* (data not shown), i.e., p27 (amino acids 261 to 280) and p25 (amino acids 241 to 260) (see also the earlier description of the dose-response experiment). Additional but weaker reactivity was detected in protein-immunized mice in response to p10 (amino acids 91 to 110), which is a region not recognized following live mycobacterial infection. Three vaccinations with 2 × 10^5 μg of plasmid DNA encoding a secreted form of Ag85A (signal sequence of human tissue plasminogen activator preceding the mature Ag85A gene) induced a 10-fold-higher spleen cell IFN-γ response following stimulation with native purified Ag85A (7,282 ± 253 pg/ml) and its synthetic peptides. IFN-γ responses in B6 mice vaccinated with Ag85A DNA were directed against peptides p25 and p27 but also against a peptide region spanning amino acids 71 to 120 (p8-p9-p10-p11). Whereas BCG-vaccinated B6 mice reacted more strongly against p25 than against p27 (18), this hierarchy was changed by DNA vaccination, resulting in stronger responses to p27 and p8 than to p25. Finally, DNA immunization followed by a protein boost dramatically increased the IFN-γ response to whole Ag (18,726 ± 4,622 pg/ml) and to the various peptides identified by DNA immunization. Responses were boosted against peptides strongly recognized following DNA vaccination but also against peptides that were only weakly recognized by DNA vaccination: amino acids 21 to 40, 131 to 160, 181 to 200, and 211 to 230.

**Spleen cell IL-2 production in B6 mice vaccinated with Ag85A protein, Ag85A DNA, or a DNA prime-protein boost regimen.** IL-2 responses towards Ag85A and its synthetic peptides could also be significantly increased by protein boosting (Fig. 3). Protein immunization induced only weak IL-2 responses in response to purified Ag85A protein (1,088 ± 271 cpm). The response in DNA-vaccinated mice was about fivefold higher (4,636 ± 1,021 cpm), and protein boosting resulted in a further threefold increase (13,217 ± 5,879 cpm). IL-2 levels in DNA-vaccinated mice were lower than those reported previously or in the dose-response experiment described above (Table 1), probably because of the later time point tested (2 months in this study versus 3 weeks after the last DNA immunization) (17, 37). DNA vaccination and the prime-boost regimen resulted in a broader IL-2-inducing epitopic repertoire than that of BCG vaccination. The strongest boost of IL-2 responses was observed in response to the immunodominant epitope identified following BCG vaccination, i.e., p25; the other epitopes elicited a weaker boost.

**Ab production in B6 mice vaccinated with purified Ag85A protein, Ag85A DNA, or a DNA prime-protein boost regimen.** All three immunization protocols resulted in significant Ag85A-specific Ab production, compared to mice immunized with MPL-A or the empty vector only (data not shown) (Table 2). However, profound differences were observed in Ab isotypes. Protein in MPL-A induced an exclusive IgG1 Ab response which was 10-fold higher than that in DNA-vaccinated mice. In contrast, DNA vaccination induced a strong IgG2a response which was 150-fold higher than that in protein-vaccinated mice.

**TABLE 2.** Ag85A-specific Ab production in B6 mice vaccinated with DNA and boosted with purified protein

<table>
<thead>
<tr>
<th>Vaccine used</th>
<th>Conc (ng/ml) of total Ig^a^</th>
<th>IgG1 (arbitrary units/ml)^b^</th>
<th>IgG2a (arbitrary units/ml)^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPL-A</td>
<td>2.34 ± 0.08</td>
<td>1 ± 0.1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Ag85A protein</td>
<td>3.70 ± 0.16</td>
<td>404 ± 17</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Ag85A DNA</td>
<td>4.40 ± 0.13</td>
<td>44 ± 1.26</td>
<td>151 ± 38</td>
</tr>
<tr>
<td>DNA-protein</td>
<td>4.71 ± 0.26</td>
<td>72 ± 4</td>
<td>350 ± 61</td>
</tr>
</tbody>
</table>

^a^ Ag85A-specific IgG concentration (mean ± SD of five sera tested individually) expressed in log_2 values.

^b^ Antibody isotype titers (determined on pooled sera) compared to a standard serum with an assigned titer of 1,000 for both isotypes.
TABLE 3. Bacterial replication in lungs from B6 mice vaccinated with Ag85A protein, Ag85A DNA, or a DNA prime-protein boost regimen and challenged with M. tuberculosis H37Rv 2 months after the last immunization

<table>
<thead>
<tr>
<th>Vaccine used</th>
<th>Level of replication (no. of CFU/lung) after iv. M. tuberculosis challengea (no. of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 days</td>
</tr>
<tr>
<td>MPL-A-control DNA</td>
<td>5.27 ± 0.20 (10)</td>
</tr>
<tr>
<td>BCG</td>
<td>3.70 ± 0.22b (3)</td>
</tr>
<tr>
<td>Ag85A protein</td>
<td>5.15 ± 0.26 (4)</td>
</tr>
<tr>
<td>Ag85A DNA</td>
<td>4.82 ± 0.27b (5)</td>
</tr>
<tr>
<td>DNA-protein</td>
<td>4.54 ± 0.16b (5)</td>
</tr>
</tbody>
</table>

a Mean ± SD (log_{10} values) is given for numbers of CFU/lung.
b P < 0.005.
c ND, not done.

**DISCUSSION**

Although DNA prime-protein boost immunization protocols are well known for their capacity to increase Ab production, much less is known concerning their effects on cell-mediated immune responses, essential in protection against intracellular pathogens such as M. tuberculosis. Here we have shown that a boost injection of protein in mice that were given a DNA vaccine encoding the mycoloyltransferase Ag85 from M. tuberculosis is capable of dramatically enhancing the Th1-type immune response primed with this DNA vaccine. Antigen-specific IL-2 and IFN-γ production in spleen cell cultures was augmented two- to fourfold by the protein boost compared to that for DNA vaccination, whereas immunization with protein in MPL-A induced only marginal levels of these Th1 cytokines, highlighting the power of DNA vaccines as priming agents for Th1-biased immune responses. Furthermore, flow cytometry analysis demonstrated that IFN-γ response was more sustained in spleen cell cultures from DNA-primed-protein-boosted mice: significant intracellular cytokine staining could be visualized up to 3 days after the onset of in vitro antigenic stimulation of CD4+ T cells from protein-boosted mice, whereas CD4+ T cells from DNA-immunized mice stained positive for IFN-γ only on day 1 of culture. Whether this sustained IFN-γ production is a mere consequence of a quantitative increase in effector cells in the protein-boosted group or the result of a qualitative difference in susceptibility to apoptosis is not clear for the moment. Analysis of Ab isotypes also showed that protein boosting following DNA priming preferentially stimulated the Th1 arm of the immune response. In complete agreement with our findings, H. M. Vordermeier et al. have recently reported on enhanced and fine-tuned immune responses by recombinant protein boosting in cattle immunized with a DNA vaccine encoding another mycobacterial antigen, i.e., HSP65 (H. M. Vordermeier, D. Lowrie, M. Singh, and R. G. Hewinson, submitted for publication).

It has previously been demonstrated that vaccination of BALB/c mice with Ag85A DNA stimulates a broader T-cell epitope repertoire than does vaccination with live BCG or infection with M. tuberculosis (6). As shown here, Ag85A DNA vaccination of B6 mice also increased this Th1-type epitopic repertoire and besides a response to the carboxy-terminal peptides p25 and p27 immunodominant in mycobacterial infection (18), an additional peptide region spanning amino acids 71 to 116 was also observed in the DNA-primed-protein-boosted mice.

TABLE 4. Bacterial replication in lungs from B6 mice vaccinated with Ag85B DNA; boosted with recombinant Ag85B protein in SBAS2A adjuvant, and challenged with bioluminescent M. tuberculosis H37Rv

<table>
<thead>
<tr>
<th>Vaccine used</th>
<th>No. of CFU/lungb (no. of mice)</th>
<th>No. of RLU/lungb (no. of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DNA</td>
<td>5.09 ± 0.21 (6)</td>
<td>4.02 ± 0.28 (6)</td>
</tr>
<tr>
<td>BCG</td>
<td>3.98 ± 0.39 (5)</td>
<td>3.15 ± 0.24 (5)</td>
</tr>
<tr>
<td>Ag85B DNA</td>
<td>4.50 ± 0.14 (4)</td>
<td>3.43 ± 0.20 (4)</td>
</tr>
<tr>
<td>DNA-protein</td>
<td>3.94 ± 0.26 (5)</td>
<td>3.13 ± 0.11 (5)</td>
</tr>
</tbody>
</table>

a Mean number of CFU ± SD (log_{10} values) as determined by plating on Middlebrook 7H11 agar.
b Mean number of relative light units (RLU) ± SD (log_{10} values) as determined in a Turner Design luminometer (15-s integration time).
thought to escape from the phagosome to the cytoplasm of the A*0201-restricted CD8+ T cells. Upon DNA vaccination, the other hand, both complete but also truncated or unfolded forms of the protein might be processed by antigen-presenting cells.

Whereas in BALB/c mice, part of the broadening of the IFN-γ repertoire by DNA vaccination is related to the induction of Ag85A-specific major histocompatibility complex class I (MHC-I)-restricted CD8+ cytotoxic T lymphocytes (CTL) (9), in Ag85A DNA-vaccinated B6 mice, cellular immune responses appear to be exclusively mediated by MHC-II-restricted CD4+ T cells. So far, we have been unable to visualize any Ag85A- or, for that matter, Ag85B-specific CD8+ responses in H-2b haplotype mice (10), most likely because Ag85 lacks the correct epitopes that could be presented by Kβ or Dβ molecules. With progressive infection, M. tuberculosis is thought to escape from the phagosome to the cytoplasm of the infected macrophage, which may then be recognized by MHC-I-restricted CD8+ T cells. We hypothesize that waning of protective efficacy of the Ag85 DNA vaccine in B6 mice is related to this lack of available MHC-I-restricted CTL epitopes. This could explain why the prime-boost immunization had a strong enhancing effect on CD4+-mediated IFN-γ production, whereas the effects on reducing bacterial burden in lungs could be demonstrated only at early time points after challenge.

Moreover, the question remains whether mycobacterial infection overall induces a murine Ag85-specific CD8+ T-cell response; we were unable to detect any Ag85A-specific CTL response following BCG vaccination or M. tuberculosis infection even in BALB/c mice (9), although three CTL epitopes could be defined in this mouse strain following DNA vaccination. It must be mentioned that the situation may be different in humans from that in mice, as Ag85A- and Ag85B-specific CD8+ T cells have been identified in BCG-vaccinated donors, using target cells infected with recombinant vaccinia virus expressing the mycobacterial antigens (32, 33). Moreover, we have recently been able to identify Ag85B-specific HLA-A*0201-restricted CD8+ epitopes using Ag85B DNA vaccination in HLA-transgenic mice, and these epitopes were also recognized in BCG-vaccinated individuals (11).

In contrast to infections with viral and protozoal pathogens, infection with M. tuberculosis remains largely confined to an intracellular localization, mostly in the lung macrophage phagosomes, and extracellular multiplication occurs only in advanced disease. Therefore, it is generally accepted that cell-mediated immunity leading to activation of bactericidal capacity of these macrophages rather than of Abs is essential for control of the infection. Nevertheless, it cannot be excluded that Abs, particularly those present in the lung mucosa, could play some role at very early stages of infection through mechanisms of macrophage- and natural killer cell-mediated bystander cytotoxicity. Daffé and Etienne have shown that Ag85 is present in the capsule of M. tuberculosis (6) and that it is possible that antibody-dependent cell-mediated cytotoxicity mediated through Ag85-specific IgG2a immunoglobulins, preferentially induced by DNA vaccination and known for their high affinity for FcyR (23), could play some role in the initial control of TB infection. In vitro experiments are needed to confirm this hypothesis.

In conclusion, our results show that DNA priming followed by exogenous protein boosting is an effective way to increase the immunogenicity and protective efficacy of an experimental TB DNA vaccine encoding Ag85 and that this technique underlines the essential role of MHC-II-restricted Th1-type CD4+ helper T cells in the protection mediated by this vaccine.

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