DNA Vaccine Using *Mycobacterium bovis* Ag85B Antigen Induces Partial Protection against Experimental Infection in BALB/c Mice

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Bovine tuberculosis is a major cause of economic loss in countries where it is endemic, and in some countries, it may be a significant zoonotic disease problem. Therefore, new strategies for vaccine development are required, and among them, genetic immunization has potential value. The main goal of this study was to test the *Mycobacterium bovis* Ag85B gene as a DNA vaccine following challenge with an *M. bovis* virulent strain (ATCC 19274). Groups of BALB/c mice (n = 10) were immunized four times intramuscularly with the pCI-Ag85B construct or the pCI vector alone as the control. High titers of total immunoglobulin G (IgG), IgG1, and IgG2a anti-Ag85B were measured in pCI-Ag85B immunized mice when compared to the pCI control group.

Regarding cellular immunity, significant levels of gamma interferon (IFN-γ) (1,100 ± 157 pg/ml) and tumor necrosis factor alpha (650 ± 42 pg/ml) but not interleukin-4 were detected in splenocyte culture supernatants of pCI-Ag85B-vaccinated mice following stimulation with recombinant Ag85B. Further, the main source of IFN-γ is CD8+ T cells, as demonstrated by intracellular cytokine staining. As far as protection, a significant reduction in bacterial load in spleens (P < 0.05) was detected in pCI-Ag85B-immunized mice compared to the pCI vector control group. The results obtained here suggest that use of the Ag85B DNA vaccine is a promising strategy to control *M. bovis* infection due to its ability to induce a Th1 type of immune response. However, protective efficacy needs to be improved, since partial protection was achieved in spleens but not in lungs of vaccinated mice.
prime/boost strategies (18, 27). Our data suggest that the DNA vaccine carrying the Ag85B gene induced high levels of anti-Ag85B antibodies, a Th1 type of immune response with significant production of gamma interferon (INF-γ) and tumor necrosis factor alpha (TNF-α) and low levels of interleukin-4 (IL-4) in BALB/c mice, and partial protection in immunized animals.

MATERIALS AND METHODS

Subcloning of the Ag85B gene. The Ag85B gene of M. bovis was amplified by PCR and subcloned into the mammalian expression vector pCI (Promega Corp., Madison, WI). Primers containing one artificial restriction site at each end were constructed according to the Ag85B gene nucleotide sequence (GenBank accession number X62979). The primer sequences were (forward) 5'-GGCAATCTTCCTCCGGGCGGGCTG-3' (EcoRI) and (reverse) 5'-GCTCTAGATCAGCCGGCGCTAAACG-3' (XbaI). PCR was performed in a 10-μl volume containing 1 ng DNA template (pMAL-Ag85B, previously constructed in our laboratory), 5 pmol of each primer, 10 mM deoxyribonucleoside triphosphate, 10 mM MgCl2, 200 μM of each dNTP, and 5 U AmpliTaq polymerase. PCR amplification was conducted with a DNA thermal cycler under the following conditions: denaturation at 95°C for 30 s, annealing at 54°C for 45 s and extension at 72°C for 1 min (30 cycles). The amplified products were purified with the QiAex II gel extraction kit (QIAGEN, Valencia, CA) and digested with EcoRI and XbaI restriction endonucleases (Invitrogen). After digestion, the PCR product was purified again by the QiAex II gel extraction kit and reconstituted to the previously PCR vector. This construction was used to transform Escherichia coli DH5α, and a single recombinant clone was selected. Plasmid DNA was extracted with the Wizard Miniprep kit (Promega Corp., Madison, WI). The pCI-Ag85B construct was digested with endonuclease and DNA sequence confirmed to the presence and the orientation of Mycobacterium Ag85B gene.

DNA sequence analysis. Double-stranded DNA sequencing of the M. bovis Ag85B gene inserted into pCI vector (Promega) was performed using the DyeNamic ET dye terminator cycle sequencing kit (MegaBACE) and the MegaBACE 1000 capillary sequencer (Amersham Biosciences, São Paulo, Brazil). The sequence data were compiled and analyzed with the sequence analysis program DNASIS V5.00 (Hitachi Software). Subsequent homology searches were performed with the BLAST programs available from the National Biotechnology Information Center (2).

Ag85B DNA vaccine. DNA vaccine construct pCI-Ag85B was amplified in the E. coli DH5α strain, and DNA isolation was performed using the EndoFree Plasmid Giga kit (QIAGEN, Valencia, CA). DNA was resuspended in phosphate-buffered saline (PBS) at a final concentration of 1 mg/ml for further immunization. The plasmid preparation contained less than 0.05 endonuclease units per 100 μg of DNA, as assessed by the QCL-1000 LumaI ameba lysis analysis kit (BioWhittaker, Walkersville, MD). The relative amount (%) of supercoiled DNA isolated in each vaccine sample was determined using densitometric analysis and reference plasmid preparations for calibration. The proportion of supercoiled isoform in the vaccinal sample was on average 70%.

Production of recombinant Ag85B protein. Escherichia coli harboring the pMAL-Ag85B construct was cultured in LB medium, and expression of the fusion protein was induced by 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After IPTG induction, bacterial cells were harvested by centrifugation at 4,000 × g for 20 min, and the supernatants were discarded. The pellets were resuspended in 100 ml of PBS (pH 8.4) containing 25 mg of lysozyme. The resuspended pellets were frozen and thawed three times at −70°C. The resulting suspensions were sonicated twice for 30 s and then centrifuged at 9,000 × g for 45 min. The protein present in the bacterial supernatant was located onto a polyacrylamide gel to confirm the presence of the recombinant protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). E. coli lysates containing the induced maltose binding protein (MBP)-Ag85B fusion protein were diluted 1:5 in PBS. Then, the suspensions were loaded into an amlyose resin column (New England Biolabs) and washed 10 times with PBS. The fusion protein concentration was determined by a Bradford protein assay.

SDS-PAGE and immunoblotting. E. coli extracts expressing the MBP-Ag85B fusion protein and purified MBP-Ag85B protein were separated on a 10% denaturing polyacrylamide gel by electrophoresis. From the gel, the proteins were transferred to nitrocellulose membranes (Bio-Rad) by Western blotting as previously described (20). The nitrocellulose membranes were blocked with 5% skim milk at 4°C overnight and reacted with rabbit anti-MBP serum (1:10,000) for 2 h at room temperature. After a reaction with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl–0.02 M Tris [pH 7.5], 0.05% Tween 20) and incubated for 1 h at room temperature with anti-rabbit immunoglobulin (IgG)-alkaline phosphatase conjugate (1:10,000) (Promega). After three washes with TBST, the reaction mixture was developed following incubation at room temperature with nitroblue tetrazolium chloride and BCIP (5-bromo-4-chloro-3-indoly-1-phosphate) for alkaline phosphatase.

Animals and immunization protocol. Forty BALB/c mice, on average 8 weeks old, were divided into groups of 10 mice each. For intramuscular (i.m.) DNA immunization, mice were preinjected with 10 μm cardiotoxin (Sigma) 5 days before vaccination. The cardiotoxin was given to optimize the immune response by inducing regeneration in muscle cells and DNA uptake as previously described (13). At days 0, 15, 30, and 45, the construct pCI-Ag85B was administered by i.m. injection. Each quadriceps was inoculated with 50 μl of DNA at a concentration of 1 μg/μl in PBS such that each animal received a total of 100 μg of plasmid DNA. As a negative control, one group of mice was injected with pCI plasmid without an insert. Another mouse group was vaccinated with 106 CFU of BCG obtained from Fundação Ataulpho de Paiva (São Paulo, Brazil) and injected subcutaneously. As a negative control of BCG immunization, PBS was administered subcutaneously to another mouse group.

Analysis of antibody response. Mice were bled 15 days after each immunization, and individual mouse sera were tested for antibody responses by enzyme-linked immunosorbent assay (ELISA). Plates (96-well) (Maxisorp; Nunc) were incubated overnight with the recombinant Ag85B protein (5 μg/ml) in carbonate-bicarbonate buffer, pH 9.6, at 4°C. Then, blocking solution (PBS containing Tween 20 [0.05%] plus 10% fetal bovine serum) was added for 2 h at 37°C. Sera from immunized mice were diluted 1:100 in PBS-Tween 20, added to the plates and incubated for 2 h at 37°C. To determine total serum IgG, IgG1, or IgG2a, plates were treated with a goat anti-mouse IgG (Sigma) or peroxidase-conjugated goat anti-mouse IgG (1:10,000; Sigma) and anti-ovine IgG (1:6,000; Sigma), respectively. The reaction mixture was developed by the addition of 200 μmol of o-phenylenediamine (Sigma) and 0.04% H2O2. The reaction was stopped by the addition of 5% H2SO4, and plates were then read at 492 nm in an ELISA reader (Bio-Rad).

Cytokine assay. Splenocytes of mice 2 weeks after the last immunization were passed through a 70-μm mesh to obtain single-cell suspensions. Splenocytes were then isolated by density gradient centrifugation with Ficoll (Sigma). Cells were washed twice with sterile PBS then cultured in RPMI 1640 medium supplemented with penicillin G-sodium (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (250 ng/ml), and 10% fetal bovine serum and then placed at 1 × 106 cells/well in 96-well tissue culture plates. Murine splenocytes from vaccinated animals were stimulated with recombinant Ag85B protein at 25 μg/ml. Nonstimulated splenocytes were used as a negative control, and splenocytes stimulated with concanavalin A (5 μg/ml) were used as a T-cell-activating control. After 72 h at 37°C in air with 5% CO2, splenocytes were incubated with recombinant Ag85B protein at 25 μg/ml. Nonstimulated splenocytes were used as a T-cell-activating control. After 72 h at 37°C in air with 5% CO2, splenocytes were cultured in medium alone or stimulated with Ag85B (50 μg/ml) or ConA (5 μg/ml). After 16 h of culture, 1 μl/μl of brefeldin A (1-mg/ml stock) (Sigma Chemical Co., St. Louis, MO) was added to impair cytokine secretion. After 4 h of incubation, these cells were stained for surface markers and intracellular cytokines as described by Bottrel et al. (4).

Intracellular cytokine staining. For intracytoplasmatic cytokine staining, splenocytes from five mice/group were pooled and adjusted to 2 × 106 cells per ml. Splenocytes were stained for surface markers and intracellular cytokines as described by Bottrel et al. (4). Briefly, cultured cells were stained for surface markers using fluorescein isothiocyanate-labeled anti-CD4 (Serotec, Düsseldorf, Germany) and anti-CD8 (Serotec) monoclonal antibodies by incubation for 20 min with antibody solution (0.15 M PBS, 0.5% bovine serum albumin, 2 mM NaN3), followed by washes and fixation using 2% formaldehyde solution. These cells were permeabilized and further stained with phycoerythrin-labeled anti-IFN-γ (Serotec) and anti-TNF-α (Serotec) monoclonal antibodies in a 0.5% saponin solution in PBS. After 15 min, cells were washed with permeabilization solution and resuspended in PBS. A minimum of 30,000 splenocyte-gated events were acquired in list mode and analyzed using a lymphocyte gate determined based on size and granularity profiles. All quadrants were set according to the labeled isotype controls and analyzed using the CELLQuest software (Becton Dickinson, San Jose, CA).

M. bovis challenge. Two weeks after the fourth DNA immunization, mice were infected intravenously with 1 × 106 CFU of virulent M. bovis (ATCC 19274). Animals were then killed 2 weeks after challenge infection. Splencs and lungs from infected and uninfected control mice were homogenized in PBS, 10-fold serially diluted, and plated on Lowenstein-Jensen medium. Plates were incubated at 37°C in air with 5% CO2, and the number of CFU was counted visually after 21 days.

Statistical analysis. Statistical analysis was performed with Student’s t test using the MINITAB software package (Minitab, State College, PA). For chal-
challenge studies, a mean value for each spleen and lung count was obtained after log conversion. Log units of protection were obtained by subtracting the mean CFU for BCG- or DNA-immunized mouse groups from the mean CFU for the corresponding control groups that received PBS or plasmid control alone, respectively.

RESULTS

Mycobacterium Ag85B protein production. The heterologous expression of M. bovis Ag85B protein was performed in E. coli as an MBP fusion. Recombinant Ag85B was purified by affinity chromatography on an amylose resin column (Fig. 1A). Purified rAg85B was analyzed by immunoblotting using rabbit anti-serum to MBP (Fig. 1B). The observed molecular mass of the recombinant protein was in accordance with the estimated molecular mass of the MBP fusion, 72 kDa (30 kDa for Ag85B and 42 kDa for MBP). The immunoblotting analysis confirmed the identity of the MBP-Ag85B fusion protein produced through rabbit anti-MBP antibody recognition.

Kinetics of total IgG anti-Ag85B production. In order to determine the level of total IgG induced after i.m. gene immunization by the pCI-Ag85B plasmid, blood samples were

FIG. 1. (A) Coomassie blue-stained SDS–10% PAGE profile of E. coli extracts expressing the MBP-Ag85B fusion protein (lane 1), or purified MBP-Ag85B fusion protein (lane 2). (B) Western blot analysis showing MBP-Ag85B recognition by rabbit anti-MBP antibodies (lane 3). The arrow indicates the MBP-Ag85B band. The numbers at the left indicate the molecular mass standard (MW) in kilodaltons (Amersham Biosciences).

FIG. 2. Total anti-Ag85B-specific IgG responses of mice immunized with DNA vaccine. Animals (n = 10) were injected i.m. with DNA vaccine construct pCI-Ag85B, or control plasmid pCI. DNA immunization was performed at days 0, 15, 30, and 45, and specific IgG levels to Ag85B were assayed by ELISA at days 15, 30, 45, and 60 after each vaccination in a working dilution of 1:100. Results are expressed as means of each group immunized. Error bars indicate standard deviations of the means. Statistically significant differences to pCI control group are denoted by an asterisk (P < 0.05). OD, optical density.

FIG. 3. Antibody subclass response in mice following i.m. immunization with the Ag85B gene. IgG1 and IgG2a subclass levels in sera of mice (n = 10) immunized with DNA vaccine pCI-Ag85B or control plasmid pCI were determined after the third immunization (day 45). Results are expressed as means of each group immunized. Error bars indicate standard deviations of the means. Statistically significant differences to pCI control group are denoted by an asterisk (P < 0.05).

OD, optical density.
Regarding protective immunity, mice immunized with the pCI-Ag85B DNA vaccine i.m. following 72 h of rAg85B stimulation. Bars indicate the mean values of cytokines in pg/ml ± standard deviations. Statistically significant differences compared to pCI control group are denoted by an asterisk (*, P < 0.05).

Levels of IgG1 and IgG2a produced in BALB/c mice vaccinated with pCI-Ag85B. In order to determine the type of immune response induced, the subclasses IgG1 and IgG2a were also analyzed. Both isotypes IgG1 and IgG2a anti-Ag85B were produced at significantly higher levels in pCI-Ag85B-vaccinated mice than in pCI-vaccinated mice after the third immunization (Fig. 3).

Induction of Th1 cytokine profile following Ag85B DNA immunization. To further determine the cytokine profile after DNA vaccination, the levels of IFN-γ, TNF-α, and IL-4 in immunized mice were measured 2 weeks after the last immunization. Figure 4 shows that high levels of IFN-γ and TNF-α were detected in cell supernatants of pCI-Ag85B-vaccinated mice in vitro stimulated with rAg85B compared to pCI-vaccinated animals. Regarding IL-4, no significant levels were detected when cells from pCI-Ag85B and control groups were specifically activated. For a negative control, MBP alone was used to stimulate splenocytes, and no significant T-cell activation was detected in either mouse group (data not shown). In contrast, ConA significantly stimulated the cytokine production by splenocytes from all vaccinated mouse groups (data not shown).

CD8+ T cells are the main source of IFN-γ. To determine which cell phenotype was responsible for cytokine production, splenocytes were stained for CD4 and CD8 surface markers and for cytoplasmic IFN-γ and TNF-α. CD8+ T cells were the major source of IFN-γ in splenocyte cultures of mice immunized with pCI/Ag85B compared to pCI empty plasmid-injected animals. (Table 1).

Level of protection induced by vaccination with the Ag85B gene. Regarding protective immunity, mice immunized with BCG had a significant reduction in CFU numbers of M. bovis in both spleens (log 0.87) and lungs (log 1.56) compared to the control mice that received PBS. Moreover, Ag85B DNA immunization induced a statistically significant reduction in M. bovis CFU numbers in spleens (log 0.56) compared to what was seen for mice that received the plasmid pCI alone (P < 0.05) (Table 2). However, there was no significant reduction in bacterial load in lungs of pCI-Ag85B-immunized mice compared to the pCI control group.

**DISCUSSION**

Protective immunity against mycobacterial infection is mediated by interactions between specifically primed CD4+ and CD8 T cells and activated macrophage effector cells harboring the intracellular pathogen (14). IFN-γ is a critical cytokine that activates macrophages and plays a pivotal role in antimicrobial protection, as demonstrated with gene knockout mice (10). Gene vaccines have been employed successfully in animal studies to induce protective immunity against a variety of bacteria, viruses, and parasites (1). This type of vaccine is capable of eliciting a strong cell-mediated immunity that is required to control infection by many intracellular agents, such as *Mycobacterium* spp. (23). Here, we demonstrated that DNA vaccination using a plasmid encoding *Mycobacterium bovis* Ag85B is a potent strategy to generate a specific Th1 type of immune response and partial protection against *M. bovis* infection.

More than 50 million cattle are infected with *M. bovis* worldwide yearly, and the resulting economic losses are estimated to be around US$3 billion per year (28). BCG, the attenuated vaccine strain of *M. bovis*, has been used as a vaccine against human tuberculosis (11). However, primarily due to its inter-

**TABLE 1. T-cell subpopulation producing IFN-γ or TNF-α following DNA immunization with pCI-Ag85B**

<table>
<thead>
<tr>
<th>Cell subpopulation</th>
<th>Cytokine</th>
<th>% Cytokine-producing cells</th>
<th>Nonstimulated</th>
<th>rAg85B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>IFN-γ</td>
<td>0.50 ± 0.16</td>
<td>1.0 ± 0.18*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>0.14 ± 0.05</td>
<td>0.84 ± 0.10*</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>IFN-γ</td>
<td>0.22 ± 0.08</td>
<td>2.2 ± 0.35*#</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>0.10 ± 0.06</td>
<td>0.40 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*a*, statistically significant difference compared to nonstimulated cells; #, statistically significant difference compared to CD4+ T cells producing IFN-γ.

**TABLE 2. Protection level induced by immunization with the Ag85B DNA vaccine compared to BCG**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log value</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU*</td>
<td>Protection</td>
<td>CFU*</td>
</tr>
<tr>
<td>PBS</td>
<td>8.12 ± 0.34</td>
<td>8.53 ± 0.15</td>
<td>8.12 ± 0.34</td>
</tr>
<tr>
<td>pCI</td>
<td>7.86 ± 0.37</td>
<td>8.56 ± 0.17</td>
<td>7.86 ± 0.37</td>
</tr>
<tr>
<td>pCI-Ag85B</td>
<td>7.83 ± 0.25</td>
<td>0.03</td>
<td>8.00 ± 0.05</td>
</tr>
<tr>
<td>BCG</td>
<td>6.53 ± 0.14</td>
<td>1.56b</td>
<td>7.66 ± 0.06</td>
</tr>
</tbody>
</table>

* Mean representative ± standard deviation of one experiment that was performed twice with similar results.

b * P < 0.05, significant compared to the PBS control.

*P* < 0.05, significant compared to the pCI control group.
ference with the intradermal tuberculin test as well as lack of protective immunity, the BCG vaccine is not used for bovine tuberculosis prevention (29). Immunization with genes encoding mycobacterial antigens such as hsp65, Ag85A, Ag85B, MPT-64, and ESAT-6 have been reported to be effective in experimental models of human tuberculosis (16, 18, 22, 30). However, few reports deal with experimental models for bovine tuberculosis using DNA vaccines. Vordermeier et al. (31), using MPT70 and MPT83 DNA vaccines in cattle, induced specific antibody and IFN-γ responses but failed to engender protective immunity against M. bovis challenge. On the other hand, DNA immunization encoding the combination of M. tuberculosis antigens Ag85B, MPT64, and MPT83 induced partial protection in cattle against M. bovis challenge (7). The Ag85 antigen complex, including Ag85A, Ag85B, and Ag85C, is recognized by T cells isolated from M. bovis-infected animals, and this complex constitutes 20 to 30% of all proteins present in supernatant of Mycobacterium short-term culture.

In this study, we analyzed both the humoral and the cell-mediated immune responses induced by the M. bovis Ag85B DNA vaccine. Mice immunized with the M. bovis Ag85B DNA vaccine mounted a strong anti-Ag85B IgG response after the third immunization compared to animals that received pCI vector alone. Regarding IgG isotypes, both IgG1 and IgG2a to Ag85B were produced in DNA-immunized mice compared to control group. As demonstrated here, Huygen et al. (16) reported that mice vaccinated with a DNA vaccine encoding Ag85A produced high levels of IgG1 and IgG2a. The protective immunity against tuberculosis depends on the recruitment of antigen-specific T cells, especially CD4+ T cells, and the release of cytokines, particularly IFN-γ, for the activation of microbicidal mechanisms of macrophages (19). Therefore, we measured IL-4, IFN-γ, and TNF-α levels from splenocyte supernatants of DNA-vaccinated mice. The results clearly indicated that the Ag85B DNA vaccine induced a Th1 type of immune response in mice, with higher levels of IFN-γ and TNF-α and no IL-4. Several studies have implicated, besides the role of IFN-γ, the role of TNF-α in the control of infections caused by Mycobacterium spp. (5, 25). Moreover, TNF-α in synergy with IFN-γ induces expression of NO82 (14). Therefore, proinflammatory cytokine synthesis and production of nitric oxide are the main effective mechanisms in fighting mycobacteria (26). Regarding the cell phenotype responsible for IFN-γ production, intracellular cytokine staining was performed, and we observed that the main source of IFN-γ is CD8+ T lymphocytes. As demonstrated by Caruso et al. (8), early production of IFN-γ by CD4+ T cells is essential to control M. tuberculosis infection, and IFN-γ production by other cells cannot substitute for the CD4 T-cell contribution. Therefore, the low efficacy of the Ag85B DNA vaccine may be due to the lower production of IFN-γ by CD4+ T cells induced by this immunogen.

Regarding protective immunity, M. bovis Ag85 DNA immunization induced a significant reduction in bacterial burden in spleens but not in lungs of vaccinated mice (Table 2). Pulmonary immunity requires activation of memory T cells producing IFN-γ with homing properties to the lung (17). Systemic immunizations are not particularly effective for inducing such pulmonary effector T cells, but combinations of intramuscular injections with mucosal plasmid instillations may overcome this problem. The major difficulty to mucosal plasmid DNA deliv-

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