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Immunogenicity and Protective Efficacy of Tuberculosis DNA Vaccines Encoding Putative Phosphate Transport Receptors¹

Audrey Tanghe,* Philippe Lefèvre,* Olivier Denis,* Sushila D'Souza,* Martine Braibant,* Evelyne Lozes,* Mahavir Singh,[†] Donna Montgomery,[‡] Jean Content,* and Kris Huygen^{2*}

Using culture filtrate Ag-specific mAbs generated from mycobacteria-infected H-2^b haplotype mice, we have previously identified three genes in the *Mycobacterium tuberculosis* genome, encoding proteins homologous to the periplasmic ATP-binding cassette phosphate-binding receptor PstS of the phosphate-specific transport system of *E. coli*. To define the potential vaccinal properties of these phosphate-binding proteins, female C57BL/6 mice were injected i.m. with plasmid DNA encoding PstS-1, PstS-2, or PstS-3 proteins from *M. tuberculosis* and immunogenicity and protective efficacy against i.v. challenge with *M. tuberculosis* H37Rv was analyzed. Significant levels of highly Ag-specific Abs and Th1-type cytokines IL-2 and IFN- γ could be detected following vaccination with each of the three genes. However, only mice vaccinated with PstS-3 DNA demonstrated significant and sustained reduction in bacterial CFU numbers in spleen and lungs for 3 mo after *M. tuberculosis* challenge, as compared with CFU counts in mice vaccinated with control DNA. Vaccination with PstS-2 DNA induced a modest reduction in CFU counts in spleen only, whereas vaccination with PstS-1 DNA was completely ineffective in reducing bacterial multiplication. In conclusion, our results indicate that DNA vaccination is a powerful and easy method for comparative screening of potentially protective Ags from *M. tuberculosis* and that the PstS-3 protein is a promising new subunit vaccine candidate. *The Journal of Immunology*, 1999, 162: 1113–1119.

Tuberculosis remains a major health problem worldwide, with an estimated annual incidence of 8 million new cases and an annual mortality of 3 million people (1). Combination chemotherapy is very effective to cure this disease, but unfortunately the treatment is long and expensive and requires stringent compliance to avoid the development of multidrug resistant forms. The only tuberculosis vaccine currently available is the attenuated strain of *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG)³. BCG continues to be widely administered to children in developing countries (World Health Organization/Expanded Program of Immunization estimate of 100 million doses each year), yet its efficacy remains controversial, particularly against the pulmonary form of the disease in young adults (2). Clearly, the development of a better vaccine could be an effective solution to the global threat of tuberculosis. As alternatives to BCG, there are several experimental approaches to tuberculosis prophylaxis, including rationally attenuated *Mycobacterium tuberculosis*, recom-

binant BCG overexpressing *M. tuberculosis* Ags, and protein subunit vaccines in appropriate Th1-type adjuvants (3, 4).

A major limitation for all these new approaches is the fact that the protective Ags for tuberculosis are still not precisely defined. As supported by observations in mice and guinea pigs, immune recognition of secreted or surface-exposed proteins of the bacillus, rather than of cytoplasmic Ags, may be an effective host strategy to control bacterial multiplication from the onset of infection. Thus, immunization with whole *M. tuberculosis* culture filtrate (CF) (rich in these exported proteins) can induce significant protection against subsequent challenge with the tubercle bacillus (5, 6). Using the technique of DNA immunization, we have recently shown that vaccination with plasmid DNA encoding a major protein component from *M. tuberculosis* CF, i.e., Ag85A (7), can indeed protect mice against low-dose aerosol challenge with *M. tuberculosis* (8). However, two-dimensional PAGE of *M. tuberculosis* CF has revealed more than 200 different protein spots (9), and thus it is very likely that protein components other than Ag85A also contribute to the protective efficacy of this CF (10).

Using CF-specific mAbs derived from BCG-vaccinated mice (11), we previously identified three genes in the *M. tuberculosis* genome encoding three putative phosphate binding proteins homologous to the periplasmic ATP-binding cassette (ABC) phosphate-binding receptor PstS from *Escherichia coli* (12–14). We have called these proteins PstS-1 (identical to the well-known 38-kDa protein also called protein Ag b (pab) (15–17) and recognized by mAb HBT-12), PstS-2 (a 37- to 38-kDa doublet protein recognized by mAb 2A1-2), and PstS-3 (a 40-kDa protein recognized by mAbs 2F8-3 and 2C1-5 and identified in crossed immunoelectrophoresis as Ag88 (18)). The three genes encoding these proteins are very similar (about 75% similarity between *pstS-1* and *pstS-2* or *pstS-3* and 94% similarity between *pstS-2* and *pstS-3*), and they are found on a continuous stretch of *M. tuberculosis* genome. At least *pstS-1* and *pstS-3* seem to be organized in two distinct

*Pasteur Institute of Brussels, Department of Virology, Brussels, Belgium; [†]Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany; and [‡]Merk Research Laboratories, West Point, PA 19486

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² Address correspondence and reprint requests to Dr. Kris Huygen, Pasteur Institute of Brussels, Mycobacterial Immunology, 642 Engelandstraat, 1180 Brussels, Belgium. E-mail address: chuygen@ben.vub.ac.be

³ Abbreviations used in this paper: BCG, *Mycobacterium bovis* Bacille Calmette-Guérin strain; B6, C57BL/6; PstS, phosphate-specific transport S; GST, glutathione S-transferase; ABC, ATP-binding cassette; CF, culture filtrate; NTM, nontuberculous mycobacteria; tPA, tissue plasminogen activator.

operons encoding their proper transmembrane PstC and PstA transporter molecules (13). The three PstS proteins all have a lipoprotein consensus signal, and they are exposed on the cell surface of the bacillus, as demonstrated by flow cytometric analysis using PstS-specific mAbs (14). These lipoproteins are powerful B cell Ags, and the detection of Abs against PstS-1 has been reported to be a valuable tool in the serodiagnosis of tuberculosis (19). With respect to T cell immunity, previous studies have reported in detail on PstS-1 (20–23), but nothing is known so far on T cell immunogenicity or protective efficacy of PstS-2 and PstS-3.

Using the technique of DNA vaccination, we now report on a comparative analysis of immunogenicity and protective efficacy in C57BL/6 (B6) mice of the three PstS proteins. We show that vaccination with all three genes induces strong and highly Ag-specific Abs and Th1-type T cell responses. Following i.v. *M. tuberculosis* challenge, the CFU number in spleen and lungs of mice vaccinated with plasmid DNA encoding PstS-3 was significantly lower than the number of CFU in spleen and lungs from mice vaccinated with empty-vector DNA. Vaccination with PstS-2 DNA induced a modest reduction in CFU counts in spleen only, whereas vaccination with PstS-1 DNA was completely ineffective in reducing bacterial multiplication.

Materials and Methods

Plasmid construction

Ag85A-, PstS-1-, PstS-2-, and PstS-3-encoding genes of *M. tuberculosis* were amplified without their mycobacterial signal sequence from plasmid pBlueScript SK II⁺ (pBSK; Stratagene, La Jolla, CA) containing the Ag85A sequence (24), from AA71 *EcoRI* 2800-bp fragment (16), from R1 *EcoRI* 1100-bp fragment (14), and from plasmid pBSK.B2, respectively (13). PCR was performed with cycle conditions of 1 min 30 at 94°, 2 min at 50°, and 3 min at 72°. Ag85A, PstS-1, and PstS-2 were amplified with *BglIII* containing primers (sense 85A, GGAAGATCTTTCCCGGC GGGCTTGCCG; anti-sense 85A, GGAAGATCTTGTCTGTTCCGGA GCTAGGC; sense PstS-1, GGAAGATCTCGGGCTGTGGCTCGAAAC CACCGAGCG; anti-sense PstS-1, GGAAGATCTCTGTGCGGTGGTG GTCAACGAGGCT; sense PstS-2, GGAAGATCTGCGGCGGTGGCAC CAACAGCTCGTCGT; anti-sense PstS-2, GGAAGATCTACTAGGTC AAGAAATAGCATTCACC), whereas PstS-3, which contains an internal *BglIII* site, was amplified with *BamHI* containing primers (sense PstS3, CGCGGATCTCTGTGGTAAACGACACAATGTGACC; anti-sense PstS3, CGCGGATCCCGTCAACCTCAGATCAGG). Amplified DNA was digested with *BglIII* or *BamHI*, isolated on a 1% agarose gel, and extracted on Prep-A-gene (Bio-Rad, Richmond, CA). Fragments were ligated to the *BglIII*- or *BamHI*-digested and dephosphorylated V1Jns.tPA vector (25, 26), transformed into competent DH5 *E. coli* (BRL, Bethesda, MD) and plated on Luria–Bertani agar medium containing kanamycin (50 µg/ml) (Duchefa, Haarlem, The Netherlands). Recombinant plasmid DNA was amplified in *E. coli* strain DH5 and purified on two cesium chloride-ethidium bromide gradients, followed by 1-butanol, phenol/chloroform extractions and ethanol precipitation. Plasmid DNA was adjusted to a final concentration of 1 mg/ml in saline and stored at –20°C. In these plasmids, the genes are expressed under control of the promoter and intron A of the first immediate early Ag IE1 from CMV, linked to the leader signal of human tissue plasminogen activator (tPA) and followed by a polyadenylation site of the bovine growth hormone.

Mice

C57BL/6 (B6) (H-2^b) mice were purchased from Bantin and Kingman (Hull, U.K.). Female mice, 6 to 8 wk old at the start of vaccination, were used.

Vaccination

Mice were anesthetized by i.p. injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and injected i.m. three times (at 3-wk intervals) in both quadriceps with PstS-1, PstS-2, PstS-3, or control DNA (empty V1Jns-tPA vector) in saline using a 0.3-ml insulin syringe (Becton Dickinson, San Jose, CA). Mice received 100 µg of DNA at each injection (50 µl of DNA at 1 mg/ml in each hind leg). As a first control group, mice were vaccinated with plasmid DNA encoding Ag85A, for which we have previously described a role in protection against tuberculosis (8). A second

control group of mice was injected i.v. in a lateral tail vein with 10⁶ CFU of freshly prepared BCG (strain GL2) grown as a surface pellicle on synthetic Sauton medium for 14 days (27) on the same day as the first DNA injection.

M. tuberculosis challenge

Mice were rested for 2 mo after the third DNA injection or 3½ mo after BCG vaccination before being infected i.v. in a lateral tail vein with 10⁶ CFU of *M. tuberculosis* H37Rv grown as a surface pellicle on synthetic Sauton medium for 14 days and stored as a concentrated stock solution at –70° in 20% glycerol. Mice were sacrificed at different time points following challenge. Spleen and lungs from individual animals (three in each group) were homogenized in PBS supplemented with penicillin and amphotericin, and serial 10-fold dilutions were plated on 7H11 Middlebrook agar supplemented with albumin-oleic acid-dextrose-catalase-enrichment broth (Difco, Detroit, MI). Plates were incubated at 37°C in sealed plastic bags and the number of CFU was counted visually after 4–5 wk. Results are presented in mean log₁₀ CFU/spleen or lungs ± SD. The Δlog₁₀ values were calculated as follows: log₁₀ CFU in control DNA vaccinated animals – log₁₀ CFU in vaccinated animals. For statistical analysis, Student's *t* test was used. Differences were considered as statistically significant at *p* < 0.05.

Antigens

Native Ag85A protein was purified from BCG CF as described previously by sequential chromatography on phenyl-Sepharose, DEAE-Sephacel ion-exchange, and molecular sieving on Sephadex G75 (28). Purified protein derivative, BCG CF, BCG cytoplasmic extract, and whole BCG bacilli were prepared as described before (27). For the PstS proteins, the same three “amplicons” described above under *Plasmid construction* were inserted in frame with the glutathione *S*-transferase (GST) coding region into the *BamHI* site of pGex-5X-3 (Pharmacia Biotech, Piscataway, NJ) to give the PstS-1, PstS-2, and PstS-3 fusion proteins and were transformed into *E. coli* DH5α. Following induction with isopropyl β-D-thiogalactoside, the recombinant fusion proteins were purified by chromatography on a GST-purification module (Pharmacia Biotech) from 400-ml cultures as described by the manufacturer. Purity of the purified recombinant fusion proteins was examined by Coomassie staining of 15% SDS-PAGE. Control GST material was prepared in the same manner from *E. coli* transformed with pGex-5X-3 vector. Recombinant PstS-1 used for cytokine analysis was a non-GST fusion protein prepared in *E. coli* as described before (29). Whole CF from *M. tuberculosis* H37Rv, *Mycobacterium scrofulaceum*, *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii* were prepared by ammonium sulfate precipitation from 14-day-old surface pellicle grown Sauton cultures (30).

Ab analysis

Serum levels of PstS- and Ag85A-specific Abs were determined 3 wk after the last DNA injection, in individual mice, using an indirect ELISA. Briefly, microtiter plates were coated overnight at 4°C with purified PstS-1, PstS-2, PstS-3 (all in the form of recombinant GST fusion proteins), or Ag85 (in its native form) at 5 µg/ml in borate buffer. Plates were washed with PBS 0.1% Tween 20. The remaining adsorption sites were saturated for 2 h at 37°C with proteins from skim milk (5% in PBS). After washing, 100-µl volumes of serial twofold dilutions of serum (starting at 1/50) in PBS 0.1% Tween 20 were applied for 2 h at 37°C. Plates were washed, and a peroxidase-labeled rat anti-mouse κ light chain mAb (LO-MK-1, Experimental Immunology Unit, Universite Catholique de Louvain, Brussels, Belgium) was added for 2 h at 37°C. Finally, the plates were washed and developed by the addition of 100 µl of orthophenyldiamine (Sigma, St. Louis, MO), 0.4 mg/ml, diluted in citrate/phosphate buffer, pH 5.6, and H₂O₂. The reaction was stopped with 50 µl of 1 M H₂SO₄, and OD were read at 492 nm with a automatic Multiskan MCC/340 (Titertek) reader.

Four individual mice were tested in each group. Data are expressed as OD values obtained for serum dilutions 1:1600. This dilution corresponded to the end of the plateau of sera showing high reactivity, which allows the comparison of the response with sera with lower reactivity.

Cytokine production

Vaccinated mice were sacrificed 3 wk after the last DNA injection, and spleens were removed aseptically. Spleens from four mice were pooled in each group. Spleen cells were adjusted at a concentration of 4 × 10⁶ cells/ml and grown in round-bottom microwell plates (Nunc, Roskilde, Denmark) in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with glutamine, HEPES, 50 µM 2-ME, antibiotics, and 10% heat-inactivated FCS (Life Technologies) (27). A volume of 180 µl of cell

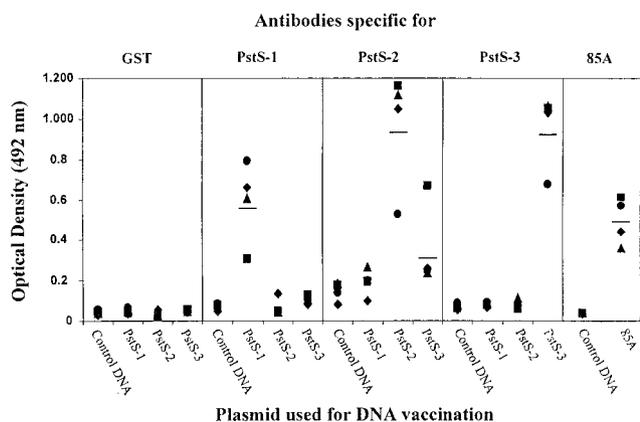


FIGURE 1. Total Abs in sera from B6 mice vaccinated with control plasmid or plasmid DNA encoding PstS-1, PstS-2, PstS-3, or Ag85A from *M. tuberculosis* and tested against *E. coli*-derived purified GST, PstS-1, PstS-2, or PstS-3 GST fusion proteins or against native Ag85A purified from BCG CF. Sera from four mice collected individually 3 wk after the third DNA injection were tested in each group. Data represent OD values measured at serum dilutions 1:1600. Horizontal bars represent mean of four values.

suspension was added to a 20 μ l of purified PstS-1, GST-PstS-2, GST-PstS-3, GST, or Ag85A (5 μ g/ml final) or the various CF (25 μ g/ml final). Cells were incubated at 37°C in a humidified CO₂ incubator, and supernatants were harvested after 24 h (IL-2) and 72 h (IFN- γ). Supernatants from three separate wells were pooled and stored frozen at -20°C until assayed. The experiment was repeated 2 wk later with two mice in each group. Finally, two mice vaccinated with PstS-3 DNA were sacrificed at week 8 after the third DNA vaccination for analysis of immune response against nontuberculous mycobacterial (NTM) CF.

IL-2 assay

IL-2 activity was measured using a CTLL-2 proliferation bio-assay. Briefly, a volume of 100 μ l of 24-h culture supernatant was added to 100 μ l of CTLL-2 cells (10^5 /ml) and incubated for 48 h (27). [³H]Thymidine (8.3 Ci/ml; Amersham, Arlington Heights, IL) was added (0.4 μ Ci/well) during the last 6 h of culture. Cells were harvested on a Titertek cell harvester (Skatron, Lier, Norway), and the radioactivity recovered on the fibermats was counted in a betaplate scintillation counter (Wallac, Turku, Finland). Each sample was tested in duplicate. Error of the assay was <10%. IL-2 levels are expressed as mean cpm. In this assay, 50,000 cpm corresponds to ~3.12 IU/ml or ~600 pg/mls and the detection limit is ~10 pg/ml (100–200 cpm) (31).

IFN- γ assay

IFN- γ activity was quantified in duplicate on 72-h culture supernatants using a mouse IFN- γ ELISA (Intertest- γ , catalogue number 80-3842-03, Genzyme, Cambridge, MA). Error of the assay was <10%. Concentrations are expressed as mean pg/ml. Detection limit in this assay is 10 pg/ml.

Results

Ab production in B6 mice vaccinated with plasmid DNA encoding PstS-1, PstS-2, or PstS-3 from *M. tuberculosis*

As shown in Fig. 1, vaccination with all three plasmid DNAs encoding PstS-1, PstS-2, or PstS-3 induced elevated Ab levels, which were highly Ag-specific, despite the fact that the three genes demonstrate significant sequence similarity. Ab response was highest in mice vaccinated with PstS-2 DNA. Abs generated by vaccination with PstS-1 and PstS-2 DNA reacted only with the corresponding proteins, one of the four mice vaccinated with PstS-3 DNA cross-reacted to some extent with PstS-2, and the other three mice reacted only to PstS-3. Confirming previous results, vaccination with plasmid DNA encoding Ag85A also stimulated a strong humoral response (8).

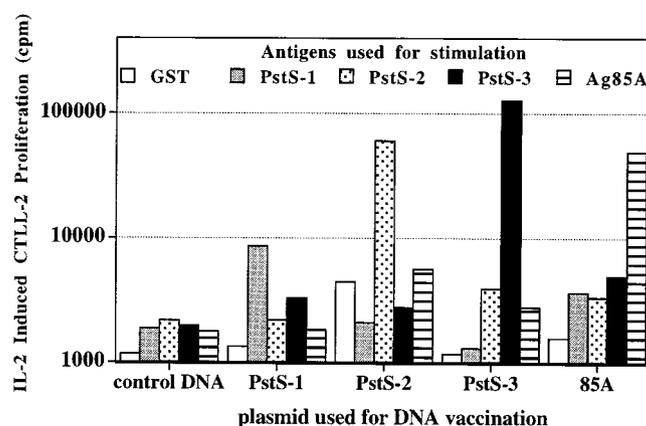


FIGURE 2. IL-2 production in 24-h culture supernatant from spleen cell cultures of B6 mice vaccinated with control DNA or DNA encoding PstS-1, PstS-2, PstS-3, or Ag85A from *M. tuberculosis* and restimulated 3 wk after the third DNA injection in vitro with the purified recombinant PstS-1, GST-PstS-2, GST-PstS-3, control GST, or purified native Ag85A from BCG CF. Splensens from four mice were pooled in each group 3 wk after the third DNA injection.

IL-2 production in spleen from B6 mice vaccinated with plasmid DNA encoding PstS-1, PstS-2, or PstS-3 from *M. tuberculosis*

Vaccination with all three plasmid DNAs induced considerable IL-2 production in spleen cell cultures stimulated in vitro with the corresponding specific Ag 3 wk after the last injection of DNA (Fig. 2). As a positive control, mice vaccinated with Ag85A DNA were also analyzed. Elevated and comparable IL-2 levels were detected in spleen cell cultures from mice vaccinated with plasmid DNA encoding PstS-2, PstS-3, and Ag85A. IL-2 levels in PstS-1 DNA-vaccinated animals were somewhat lower, whereas IL-2 levels in spleen cell cultures from mice vaccinated with control DNA were close to background levels. As for the Ab production, IL-2 production was Ag-specific and no cross-reactivity could be observed. Mice vaccinated with PstS-2 DNA demonstrated a certain degree of generalized activation reflected by IL-2 production even in unstimulated cultures or cultures stimulated with the GST control material. Significant IL-2 activity could also be measured in spleen cell cultures from PstS DNA-vaccinated mice stimulated with purified protein derivative, BCG CF, and BCG extract (data not shown). IL-2 production was also examined in spleen cell cultures from mice (two spleens pooled in each group) 5 wk after the third DNA injection. Again, vaccination with all three plasmids was effective, PstS-2 and PstS-3 being superior to PstS-1-encoding DNA (data not shown).

IFN- γ production in spleen from B6 mice vaccinated with plasmid DNA encoding PstS-1, PstS-2, or PstS-3 from *M. tuberculosis*

As for IL-2 production, all three PstS-encoding DNA constructs induced significant Ag-specific IFN- γ levels, which were highest in mice vaccinated with plasmid DNA encoding PstS-3 (Fig. 3). PstS-1 DNA also induced significant IFN- γ , whereas PstS-2 DNA generated the lowest, albeit still relatively high (about 600 pg/ml), IFN- γ response. As compared with vaccination with Ag85A DNA, IFN- γ concentration induced with PstS-3 were about two times higher. IFN- γ production was also analyzed in spleen cell cultures from mice (two spleens pooled in each group) 5 wk after the third shot of DNA, and results were similar to the ones obtained after 3 wk (data not shown).

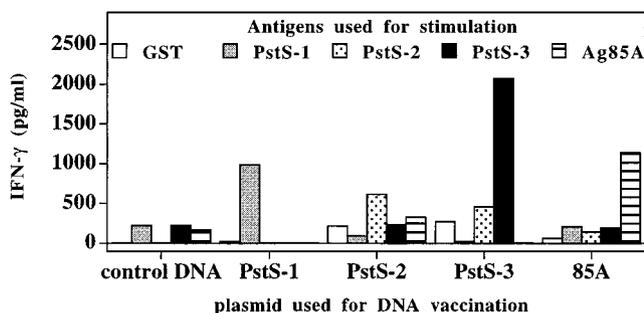


FIGURE 3. IFN- γ production in 72-h culture supernatant from spleen cell cultures of B6 mice vaccinated with control DNA or DNA encoding PstS-1, PstS-2, PstS-3, or Ag85A from *M. tuberculosis* and restimulated 3 wk after the third DNA injection in vitro with the purified recombinant PstS-1, GST-PstS-2, GST-PstS-3, control GST, or purified native Ag85A from BCG CF. Splens from four mice were pooled in each group 3 wk after the third DNA injection.

Cross-reactivity against PstS-3 from NTM

As immune responses following vaccination with the three PstS homologues appeared to be highly Ag-specific, cross-reactivity against NTM species that can be pathogenic for man was analyzed. Spleen cells from mice vaccinated with PstS-3 DNA from *M. tuberculosis* were stimulated with crude CF (as a potential source for PstS-3 protein) from four atypical mycobacterial species and were analyzed for Th1-type cytokine secretion. As shown in Fig. 4, very strong cross-reactive IL-2 and IFN- γ responses were found against CF from *M. kansasii* and particularly *M. scrofulaceum* in spleen cell cultures from B6 mice 8 wk after the third injection with PstS-3 DNA. CF from *M. intracellulare* stimulated a cross-reactive

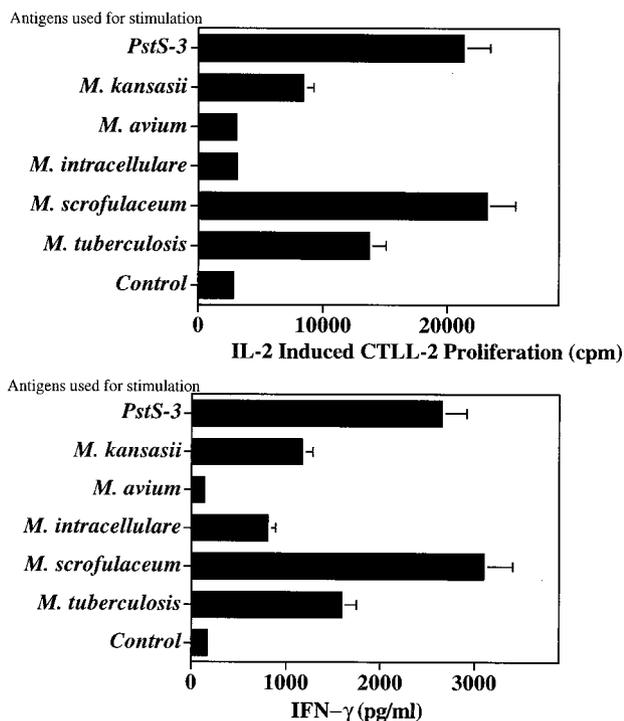


FIGURE 4. IL-2 (upper panel) and IFN- γ (lower panel) production in spleen cell culture supernatant from B6 mice vaccinated with PstS-3-encoding DNA and restimulated in vitro with purified recombinant PstS-3 (5 μ g/ml) or crude CF (25 μ g/ml) from various mycobacterial species. Splens from two mice were pooled 8 wk after the third DNA injection.

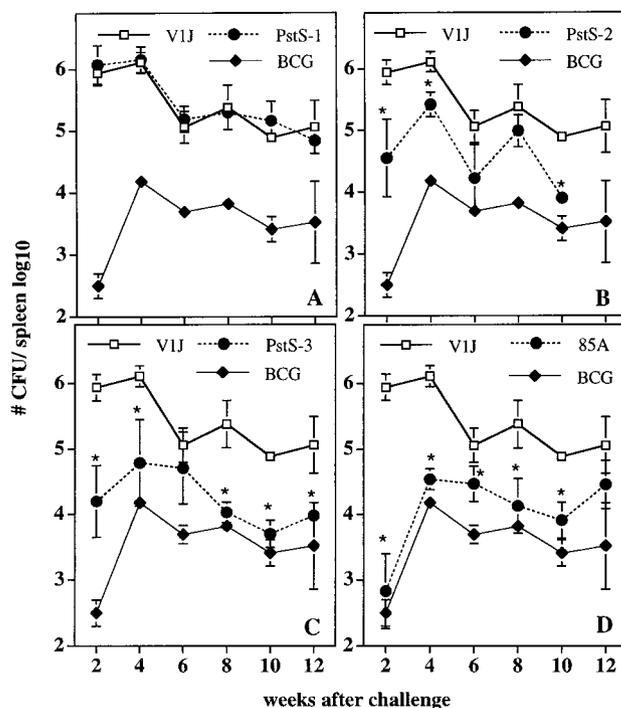


FIGURE 5. Replication of *M. tuberculosis* H37Rv in spleen from B6 mice vaccinated with control DNA (V1J), BCG, and DNA encoding PstS-1 (A), PstS-2 (B), PstS-3 (C), or Ag85A (D). Data represent the mean \log_{10} CFU \pm SD/spleen of three animals tested individually in each group. *, CFU counts from vaccinated mice are statistically different ($p < 0.05$) from CFU counts in control DNA-injected mice.

ive IFN- γ (but not IL-2) response, whereas CF from *M. avium* was not recognized at all by spleen cells from B6 mice vaccinated with PstS-3 DNA. These data suggest that immunodominant T cell epitopes on PstS-3 are highly conserved between *M. tuberculosis*, *M. kansasii*, and *M. scrofulaceum*, albeit different from the dominant T cell epitopes on PstS-1 and PstS-2. The recognition of *M. intracellulare* CF by IFN- γ -producing T cells only could suggest that this species only shares cross-reactive CD8⁺ epitope(s) with *M. tuberculosis*, but no IL-2 inducing CD4⁺ epitopes. In BALB/c mice vaccinated with 85A DNA, we have indeed found that some CD8⁺ epitopes on the Ag85A protein can induce IFN- γ secretion in the absence of IL-2 (32). Production of IL-2 in response to purified PstS-3 was lower in this experiment than in the previous one, possibly because spleens were taken at a later time point (8 wk vs 3 wk) after the third DNA vaccination. IFN- γ levels induced with PstS-3 were comparable in both experiments. Spleen cells from mice vaccinated with PstS-1 DNA showed very little cross-reactivity, whereas cells from PstS-2-vaccinated mice also reacted to some extent to CF from *M. scrofulaceum* (data not shown).

Sustained protection against *M. tuberculosis* H37Rv replication in spleen from B6 mice vaccinated with PstS-3

B6 mice vaccinated with PstS-3 (Fig. 5C) and Ag85A (Fig. 5D) DNA demonstrated significantly reduced numbers of *M. tuberculosis* CFU in their spleens as compared with mice vaccinated with control DNA. For PstS-3 DNA vaccination, the highest reductions (comparable to the ones obtained with BCG) were observed at the later time points, whereas reduction with Ag85A DNA was strongest at early time points but started to wane at 12 wk after challenge. Vaccination with PstS-1 DNA was completely ineffective (Fig. 5A), whereas PstS-2 DNA vaccine (monitored until week 10

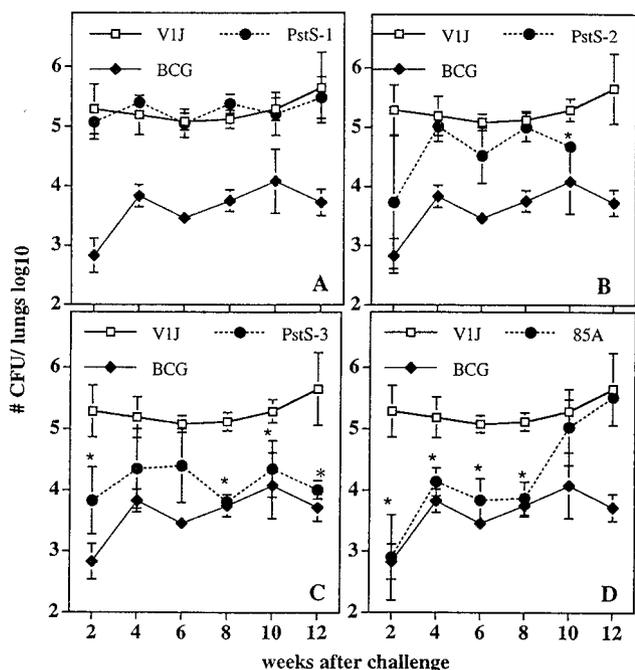


FIGURE 6. Replication of *M. tuberculosis* H37Rv in lungs from B6 mice vaccinated with control DNA (V1J), BCG, and DNA encoding PstS-1 (A), PstS-2 (B), PstS-3 (C), or Ag85A (D). Data represent the mean \log_{10} CFU \pm SD/lungs of three animals tested individually in each group. *, CFU counts from vaccinated mice are statistically different ($p < 0.05$) from CFU counts in control DNA-injected mice.

after challenge only) conferred a moderate but significant protection in spleen at weeks 4 and 10 ($\Delta\log_{10}$ 0.69 and 0.99, respectively). Comparing CFU values for the first time point, i.e., 2 wk after challenge, with those for the second time point, i.e., 4 wk, control and PstS-1 DNA-vaccinated mice demonstrated maximal CFU counts in the spleen already after 2 wk. These levels were comparable to CFU counts at week 4. CFU counts in spleen from BCG and Ag85A DNA-vaccinated mice were low at week 2 and increased about 1.5 \log_{10} during the next 2 wk. Finally, in PstS-2- and PstS-3-vaccinated mice, CFU counts in spleen were of intermediate value (between control/PstS-1 and BCG/Ag85A) at week 2 and increased 1.0 and 0.5 \log_{10} , respectively, toward weeks 4.

Sustained protection against *M. tuberculosis* H37Rv replication in lungs from B6 mice vaccinated with PstS-3

B6 mice vaccinated with PstS-3 DNA also demonstrated significant protection against *M. tuberculosis* replication in the lungs (Fig. 6C). The levels of protection were lower than those observed for BCG at week 4 and week 6 ($\Delta\log_{10} = 0.89$ for PstS-3 vs 1.36 for BCG and 0.68 vs 1.62, respectively), but were not significantly different at the later time points of 8, 10, and 12 wk of infection (1.31 vs 1.37, 0.94 vs 1.21 and 1.64 vs 1.93). Vaccination with DNA encoding PstS-1 was completely ineffective (Fig. 6A), whereas vaccination with PstS-2 DNA demonstrated a slight but significant protection at week 10 only ($\Delta\log_{10} = 0.62$, $p < 0.025$; Fig. 6B). Vaccination with plasmid DNA encoding Ag85A was also capable of reducing *M. tuberculosis* CFU values in the lungs (Fig. 6D). In contrast to the results observed for PstS-3, reductions in CFU counts in Ag85A DNA-vaccinated mice were observed during the first 8 wk of challenge but at week 10 and 12 protection CFU counts were not different from those in mice vaccinated with control DNA. As for the spleen, mice vaccinated with control or PstS-1 DNA demonstrated plateau CFU counts in the lung from

week 2 on, whereas mice vaccinated with BCG or Ag85A DNA demonstrated about a 1 \log_{10} increase between week 2 and week 4. CFU counts in PstS-2- and PstS-3-vaccinated mice were intermediate between control and BCG values at week 2 and increased to control values for PstS-2 at week 4 but only about 0.5 \log_{10} in PstS-3-vaccinated mice.

Discussion

Polynucleotide, genetic, or DNA vaccination, i.e., administration of plasmid DNA encoding microbial Ag, can result in effective protein expression in vivo, generation of humoral and cell-mediated immune responses, and protection in a variety of animal models of infectious disease including influenza, HIV, bovine herpes, rabies, malaria, leishmaniasis, herpes simplex, and cottontail papilloma (33, 34). Therefore, DNA vaccination seems to be a broadly applicable technique for generating protective immune responses against infectious pathogens without the need for live organisms or replicating vectors. DNA vaccines are easy to manufacture, they do not require protein purification (which is a major advantage when working with slow-growing organisms such as mycobacteria), and they do not require a cold chain for their storage, as they can be conserved in a dry form (35). It has been estimated that 80% of the costs for vaccines in the developing world is related to this cold storage. Finally, i.m. DNA vaccination leads to the generation of strong cellular immune responses with Th1 cytokine profile without requiring additional adjuvants. The induction of this type of immunity, by which infected macrophages are activated in their bactericidal activity by Th1 and CD8⁺ T cell cytokines such as IFN- γ and TNF- α , is thought to be essential for an effective tuberculosis vaccine (36, 37). Hence, DNA vaccination using plasmid DNA encoding *M. tuberculosis* proteins may offer a number of advantages over the classical live-attenuated or second-generation protein-subunit vaccines (38).

In recent years, it has become clear that the protective Ags for tuberculosis reside predominantly within the secreted or exported proteins of the bacillus, and early CF represent the main source of these extracellular Ags. Previously, we have shown that genes from the MHC exert a profound influence on the Ab repertoire and on the magnitude of Th1-type spleen cell cytokine secretion against these mycobacterial CF Ags (39, 40). Our studies enabled us to define two new Ags in BCG CF, a 37- to 38-kDa doublet protein and a 40-kDa protein, which were extremely immunogenic for B cells from mice with H-2^b haplotype such as B6 and MHC congenic BALB.B10 mice (40). Monoclonal Abs against these two proteins were derived from BCG-vaccinated H-2^b mice (11) and enabled the identification of two new genes in *M. tuberculosis* very similar to the previously described 38-kDa protein (also called pab, Ag78, or Ag5) (17). As all three genes code for proteins homologous to the periplasmic ABC phosphate-binding receptor PstS from *E. coli*, we have proposed to rename them as PstS-1 (38 kDa), PstS-2 (37–38 kDa doublet), and PstS-3 (40 kDa), respectively (14). In view of the essential role of phosphate in numerous biochemical processes, the strong surface expression (14), and the immunodominance of these phosphate-binding proteins for “resistant” H-2^b haplotype mice (11), we decided to compare the protective potential of these three PstS homologues using the technique of DNA vaccination.

B6 mice were vaccinated with control plasmid or with plasmid DNA encoding PstS-1, PstS-2, or PstS-3. As a positive control, mice were also vaccinated with plasmid DNA encoding the Ag85A component of the Ag85 complex, which, as previously reported, protects B6 mice against low-dose aerosol challenge given 10 wk after vaccination (8). All three PstS-encoding DNA

constructs were found to be immunogenic, as measured by Ab production or Th1-type cytokine secretion. Plasmid DNA encoding PstS-1 was the weakest of the three constructs. IL-2 and Ab titers were comparable following vaccination with DNA encoding PstS-2 and PstS-3. IFN- γ production was strongest in mice vaccinated with DNA encoding PstS-3. Despite the overall similarity between the three proteins, immune responses were Ag-specific, and very little cross-reactivity could be observed either at the B cell or at the T cell level. Protection against i.v. challenge with *M. tuberculosis* H37Rv was monitored by CFU counting over a period of 3 mo. Only vaccination with plasmid DNA encoding PstS-3 and Ag85A was found to consistently reduce the CFU counts in both spleen and lungs as compared with CFU counts in mice vaccinated with empty vector. The number of CFU was 10- to 30-fold lower in vaccinated than in control mice. Protection with Ag85A DNA was most pronounced during the first 8 wk of challenge but gradually disappeared at later time points. It may be hypothesized that expression of relevant Ag85A epitopes on the surface of the infected macrophages was down-regulated at these late time points, which may have impaired recognition by Ag85A-specific T cells, or, alternatively, that Ag85-specific T cells became exhausted through apoptotic interactions (41). In contrast, protection with PstS-3 DNA was only modest at early time points but became more pronounced later and was sustained until week 12 after challenge. To what extent relevant PstS-3-specific epitopes become more abundantly expressed at later time points on the infected macrophage is obviously a question that requires more detailed analysis.

Spleen cells from PstS-3 DNA-vaccinated mice also secreted IL-2 and IFN- γ when stimulated in vitro with whole CF from the NTM *M. scrofulaceum*, *M. kansasii*, and to a lesser extent *M. intracellulare*, suggesting that PstS-3-specific H-2^b epitopes were conserved in these three species. In 1966, Palmer and Long demonstrated that infection of guinea pigs with *M. kansasii* and *M. scrofulaceum* (Gause) could induce a BCG-like effect against virulent *M. tuberculosis*, and their results suggested that all the relevant protective Ags present in these NTM were shared with BCG (42). More recently, we have confirmed that in an experimental mouse model, BCG vaccination, and *M. scrofulaceum* infection can indeed interfere with each other, and particularly so in mice with H-2^b haplotype (30). In this context, it can be speculated that the presence of *M. scrofulaceum* in the environment could induce cross-reactive immune responses to protective Ags of *M. tuberculosis*/BCG, such as the phosphate-binding PstS-3 protein, and that these cross-reactive immune responses may partly explain the variable protection observed with BCG, particularly in tropical and subtropical regions with high incidence of environmental mycobacteria (43).

So far, protective efficacy against tuberculosis has been reported for DNA vaccines encoding hsp65 (44), the 38-kDa Ag (45), and the Ag85A component from the Ag85 complex (8). The relative potency of these DNA vaccines with respect to one another is not known. Unfortunately, it is impossible to draw conclusions from the published literature, as three different plasmid vectors, with different inherent adjuvanticity (linked to their number of unmethylated CpG-dinucleotides and "Krieg" motifs (46)), different mouse strains, different challenge models (aerosol vs i.p.), and different resting times before challenge (ranging from 2 to 10 wk) have been used. Furthermore, previous studies mostly reported on protection at one particular time point (4 wk after challenge), whereas we have monitored for the first time a challenge experiment over a 3-mo period. Variations in methodology may well explain why Zhu et al. observed protection against i.p. challenge with *M. tuberculosis* administered 2 wk after the last vaccination

with plasmid DNA encoding PstS-1 (45), whereas we were unable to find any protection following vaccination with the same *pstS-1* gene against an i.v. *M. tuberculosis* challenge after resting the mice for 2 mo. Our results clearly show that a reliable, comparative screening can only be performed when genes are compared on the same plasmid vector backbone and using the same experimental protocol.

In conclusion, vaccination with plasmid DNA encoding PstS-3 was found to be an efficient method for generating strong and sustained protective immunity in spleen and lungs following i.v. challenge with *M. tuberculosis* H37Rv in B6 mice. Preliminary results indicate that PstS-3 DNA vaccination is also effective in other mouse strains (data not shown). Obviously, these promising results in mice have to be confirmed in other animal models, such as guinea pigs and more importantly nonhuman primates, before human trials can be envisaged.

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