Vaccination of Mice and Cattle with Plasmid DNA Encoding the Mycobacterium bovis Antigen MPB83

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A scientific review of bovine tuberculosis in Great Britain has concluded that the development of a cattle vaccine holds the best prospect for long-term disease control. Recent reports of successful DNA vaccination against Mycobacterium tuberculosis in small animal models have raised the possibility of using a similar strategy to produce vaccines against Mycobacterium bovis infection in cattle. To test this possibility, BALB/c mice were immunized with DNA encoding the M. bovis antigen MPB83. The mice responded to vaccination with a mixed IgG1/IgG2a response to the antigen and were protected from intravenous challenge with virulent M. bovis to a similar extent as those vaccinated with bacille Calmette-Guérin. The immunogenicity of the DNA vaccine in cattle was tested, after having established that DNA encoding MPB83 was immunogenic and elicited protective immunity in mice. In these studies, vaccinated animals had strong proliferative responses to MPB83.

In developed countries, the introduction of pasteurization of milk and control of bovine tuberculosis by testing and slaughter-based control measures have dramatically reduced the transmission of Mycobacterium bovis infection from cattle to humans. In these countries, M. bovis is now predominantly an occupational zoonosis with a potential risk for workers in farms, abattoirs, and zoos [1–3]. In contrast, human tuberculosis caused by M. bovis is still a major health issue in many developing countries [4–7]. A number of factors maintain the threat of bovine tuberculosis to human health, including increases in the number of immunocompromised individuals [8], the emergence of strains of M. bovis resistant to known drugs, [9] and, in some countries, an increasing prevalence of disease in cattle [10].

Bovine tuberculosis also has severe implications for animal welfare, and affected farms suffer painful economic losses. In Great Britain, a recent independent scientific review for the government concluded that further control measures are needed to tackle the problem of the increasing incidence of tuberculosis in the British herd and that the best prospect for future control is to develop a vaccine to protect cattle against tuberculosis [10]. Recent advances in DNA vaccination raise the possibility of producing a vaccine against M. bovis that is at least as protective as BCG but does not compromise the tuberculin skin test [11].

To date, there have been few reports of the use of DNA immunization for livestock species such as cattle. However, in one such investigation, Cox et al. [12] immunized cattle with plasmids that encode bovine herpesvirus 1 (BHV-1) glycoproteins and reported gene-specific virus-neutralizing antibody responses that limited the shedding of BHV-1 from vaccinated calves.

In this study, we used a DNA vaccine that encodes the M. bovis antigen MPB83, which is recognized by both the humoral and cellular arms of the immune response during M. bovis infection in cattle [13, 14]. We report that BALB/c mice immunized with DNA that encode MPB83 had a mixed IgG1 and/or IgG2a response and were protected against iv challenge with virulent M. bovis to a similar extent as those vaccinated with BCG. Furthermore, cattle immunized with the DNA vaccine gave strong whole-blood proliferative responses after stimulation with recombinant MPB83 protein.

Materials and Methods

Bacterial strains. An M. bovis field strain (5260/96) was isolated from a cow in the county of Staffordshire, Great Britain, in 1996. It was propagated immediately before this study in Middlebrook 7H9 broth containing sodium pyruvate (4.16 g/L) and 0.05% (v/v) Tween-80, supplemented with 10% (v/v) Middlebrook ADC (albumin/dextrose/catalase) enrichment, at 37°C. BCG Pasteur strain was a kind gift of the Statens Serum Institut (Copenhagen). It was propagated as for M. bovis, except that 0.2% (v/v) glycerol was used in place of sodium pyruvate. Before inoculation into mice, the BCG cells were
washed, resuspended in endotoxin-free PBS, and dispersed by brief sonication with a Vibracell control unit plus CV18 converter fitted with a 3-mm-diameter tip (Sonic & Materials, Danbury, CT).

Construction and preparation of the DNA vaccines. The plasmid pCMV-83 was made by inserting the mpt83 gene (with a gene sequence identical to that of mpb83 from M. bovis [15]) from M. tuberculosis H37Rv into the plasmid backbone pCMV4. The mpt83 gene was obtained as a 700-bp product by PCR on genomic DNA from M. tuberculosis H37Rv, with use of 5′-ATTGGATCCGCTATGATCAACGTTCAG-3′ as the forward primer and 5′-TATGCGGCCGCGAAGTTACTGTT-3′ as the reverse primer. The underlined regions are BamHI and NotI restriction sites used for cloning into pCMV4 (CMV, cytomegalovirus); pCMV4 is based on the plasmid pCDNA3.1 from Invitrogen (Leek, the Netherlands), with the addition into the HindIII restriction site of the intron A from the human CMV immediate-early gene [16]. The control plasmid pCMV-link was made by removing mpb83 from pCMV-83 with use of XhoI and BamHI and by replacing it with a polyclinker containing BamHI, EcoRI, PsiI, HindIII, and XhoI restriction sites.

DNA for immunization was prepared with use of a QIA-GEN-tip 10000 plasmid extraction kit and endotoxin-free buffers (Qiagen, Dorking, UK), according to the manufacturer’s instructions. The DNA was finally resuspended in endotoxin-free PBS (Sigma, Poole, UK) before injection.

Vaccination of mice. Groups of 5 6-week-old specific-pathogen-free BALB/c mice (Harlan, Bicester, UK) were immunized with BCG Pasteur, pCMV-link, or pCMV-83 DNA. BCG-immunized mice received a single 50-μl inoculum of each hind leg (100 μg) at the beginning of the experiment, representing a vaccination dose of $1.75 \times 10^7$ cfu. DNA-immunized mice received a 50-μl inoculum (50 μg) in the quadriceps muscle of each hind leg (100 μg total) at the beginning of the experiment and on 3 subsequent occasions, 3 weeks apart.

M. bovis challenge in mice. In order to set up the M. bovis challenge model, mice were challenged iv with 10$^7$, 10$^4$, or 10$^3$ cfu of M. bovis. For subsequent vaccination and challenge studies, all mice were challenged iv with 10$^3$ cfu of M. bovis (strain 5260/96) 45 days after the final DNA immunization. Mice were killed 7 weeks after infection with M. bovis, and the lungs and spleen were removed to sterile water. The tissues were homogenized with use of a rotating-blade macerator system. Viable counts were performed on the macerate by serial dilution in sterile distilled water and plating of dilutions onto Middlebrook 7H10 agar (Difco, Detroit) containing sodium pyruvate (4.16 g/L) and 10% (v/v) Middlebrook oleate/ADC enrichment. Plates were incubated at 37°C and were examined after 6 weeks for growth of M. bovis. The number of colonies on each plate containing from 30 to 300 colonies was determined and recorded. The Mann-Whitney U test was used for all subsequent statistical analyses.

Vaccination of cattle. Three female calves of ~6 months of age were immunized with either pCMV-83 or pCMV-link. Each group of animals received 1 mg of DNA in the neck muscle on 3 occasions, 3 weeks apart.

Antigens. Bovine tuberculin (PPD-M) was obtained from the Tuberculin Production Unit at Veterinary Laboratories Agency Weybridge (Surrey, UK) and was used in culture at a concentration of 10 μg/mL. The native glycosylated form of MPB83 was purified from M. bovis AN5 by ConA affinity chromatography, as described elsewhere [17]. Unglycosylated recombinant MPB83 was obtained by cloning the gene that encodes MPB83 from M. bovis into the expression vector pET21d. By use of this vector, MPB83 was expressed in Escherichia coli as a histidine-tagged fusion protein of 28 kDa (figure 1) and purified using Ni-affinity chromatography, as described by the manufacturer (Novagen, Madison, WI). The purity of recombinant MPB83 was confirmed with a polyclonal rabbit antiserum to E. coli proteins (Dako, High Wycombe, UK; data not shown).

Figure 1 shows both recombinant and native forms of MPB83 by SDS PAGE and their recognition by monoclonal antibody SB10 (MAB M1; Agen Biomedical, Acacia Ridge, Australia). SB10 is specific for MPB83 and the closely related M. bovis antigen MPB70 [15]. The native glycoprotein MPB83 migrated to a position of 25 kDa [15] and was recognized by SB10. The other band of higher molecular weight (~29 kDa) visible by SDS PAGE is ConA monomer, an unavoidable contaminant resulting from the final ConA-purification step (data not shown).

The native form of MPB83 was used in indirect ELISA to ensure that all native conformational epitopes would be available for recognition. However, native MPB83 could not be used...
for whole-blood proliferation since, after purification, traces of detergent and ConA remained in the sample. For this reason, the affinity-purified recombinant MPB83 was used.

**Indirect ELISA.** Blood was collected from mice in all groups 2 weeks after each DNA immunization by tail bleeding. The serum from the animals in each group was pooled and stored at −20°C. At the end of the experiment, all samples were tested by indirect ELISA, to determine the IgG1 and IgG2a antibody response of the animals to MPB83 antigen. Plates (96-well Maxisorp; Nalgene Nunc International, Roskilde, Denmark) were coated with native MPB83 antigen at a 0.5-

![Figure 2. Serological response of BALB/c mice to immunization with pCMV-83 ( ), pCMV-link (△), and BCG Pasteur (○).](cid20030_suppl3_f2.jpg)

This is consistent with the low-level production of MPB83 by this strain of BCG [18].

**Protection against *M. bovis* challenge in mice.** The mouse model for infection with *M. bovis* was established previously by titrating doses of *M. bovis*. Initially, 10 strains of mice were infected with 2 different field isolates of *M. bovis* at a dose of 10^5 cfu. Both field isolates of *M. bovis* were considerably more virulent than reported for an equivalent dose of virulent strains of *M. tuberculosis*, including Erdman, H37Rv, and a clinical isolate [19–22]. Most mice died from each strain before 3 weeks. Postmortem examination revealed severe tuberculosis affecting the lungs, spleen, liver, and kidney. Greatest survival was seen among BALB/c and MRL/Lpr mutant mice (data not shown). Subsequent studies to refine the challenge model involved the use of BALB/c mice and iv doses of 10^4 and 10^3 cfu *M. bovis*. At these doses, all mice survived for 7 weeks and had tuberculosis at the postmortem examination.

With use of a challenge dose of 10^4 cfu, BALB/c mice vaccinated with BCG Pasteur had significantly less *M. bovis* 5260/96 in their lungs and spleen than did the pCMV-link control group (*P* < .05 and *P* < .01, respectively; figure 3). Significant protection against virulent *M. bovis* was observed in the lungs and spleens of mice immunized with pCMV-83 (*P* < .01 for both organs). Furthermore, immunization with pCMV-83 gave significantly more protection against *M. bovis* in the spleen than did immunization with BCG (*P* < .05). This was not the case for protection against *M. bovis* in the lung.

**Immune responses to DNA vaccination in cattle.** Having established that DNA encoding MPB83 is both immunogenic and able to elicit protective immunity in a small animal model of infection, we tested the immunogenicity of the DNA in cattle.
Eight weeks after the last immunization, the calves were bled, and the blood was used in a whole-blood proliferation assay with either recombinant MPB83 protein or PPD-M as the antigen. Figure 4 shows the mean SIs obtained for each group of calves after in vitro stimulation with MPB83 or PPD-M at 10 μg/mL. Immunization of cattle with pCMV-83, but not pCMV-link control DNA, resulted in significant proliferative responses after stimulation with either antigen. The response to PPD-M was strong (4 times the pCMV-link response) but less than that to recombinant MPB83 protein.

Discussion

A number of strategies have been proposed for the development of improved tuberculosis vaccines. These include the development of rationally attenuated strains of *M. bovis* or *M. tuberculosis*, as well as the use of protein subunit and DNA vaccines [10, 23]. DNA vaccination constitutes an exciting new strategy for the development of nonliving subunit vaccines because of the generic nature of the production and purification processes required. Several mycobacterial antigens have been defined as protective against *M. tuberculosis* when delivered as DNA vaccine to small animals (e.g., [24±26]). However, this report is, to our knowledge, the first to describe DNA vaccination that confers protection against *M. bovis* infection.

There have been several reports that *M. bovis* is more virulent than *M. tuberculosis* for mice ([27] and reviewed in [28]). Therefore, the parameters of a mouse challenge model were established for field isolates of *M. bovis* before any vaccination-challenge experiments were performed. Consistent with findings in studies by Dunn and North [27], *M. bovis* was more virulent for the mice in our study than was *M. tuberculosis*, causing death through acute tuberculosis. To avoid this, we had to use 4 logarithms less of a field isolate of *M. bovis* in BALB/c mice than a similar study that used a clinical isolate of *M. tuberculosis* [20].

MPB83 was chosen for our vaccination studies since it is a dominant antigen during infection in 2 of the natural target hosts for *M. bovis* in Great Britain (i.e., cattle [13, 14] and badgers [17]). The results reported here demonstrate that DNA vaccination with this antigen can confer significant protection against severe challenge with an *M. bovis* field strain in mice, especially in the spleen. Previous studies have shown that when DNA vaccination protects mice against subsequent challenge with *M. tuberculosis* [24–26], it does so by establishing a cellular immune response that is dominated by antigen-specific T lymphocytes that both produce IFN-γ and are cytotoxic toward infected cells (a type 1 cellular immune response). The immune response stimulated by DNA vaccination with mpb83 in mice was a mixed IgG1/IgG2a response. However, the exact nature of the immune response that confers protection against *M. bovis* infection remains to be elucidated. It is encouraging that strong T cell responses were observed after DNA immunization with mpb83 in cattle.

In Great Britain, tuberculosis in cattle is controlled by a test-and-slaughter strategy that relies on the tuberculin skin test to identify cattle infected with *M. bovis*. In order to continue with this strategy, it is essential to develop vaccines that do not compromise diagnosis [10]. Such vaccines would also be beneficial for use in humans, to enable identification of individuals who, despite vaccination, contract tuberculosis and require chemotherapy. Recently it has been demonstrated that DNA vacc-
cination of guinea pigs with Ag85A resulted in protective immunity without sensitizing the animals to the tuberculin skin test [11]. In this study, whole blood taken from mpb83-vaccinated calves responded to PPD-M in vitro. However, whether DNA vaccination with mpb83 compromises tuberculin skin testing in cattle remains to be determined.

In conclusion, our results have demonstrated that DNA vaccination with mpb83 protects mice against challenge with M. bovis and stimulates strong proliferative responses in cattle. Experiments are now in progress to determine the protective efficacy of this vaccine against pulmonary challenge with low doses of M. bovis in guinea pigs and cattle.

References


