Effective, Nonsensitizing Vaccination with Culture Filtrate Proteins against Virulent *Mycobacterium bovis* Infections in Mice

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Received 14 January 1998/Returned for modification 23 March 1998/Accepted 7 July 1998

Vaccination of mice with *Mycobacterium bovis* culture filtrate proteins (CFP), prepared in a variety of adjuvants (aluminum hydroxide, Quil-A, and dimethyldioctadecyl ammonium bromide [DDA]), provided significant protection against an aerosol challenge of virulent *M. bovis*. Additionally, vaccination with CFP in DDA or Quil-A did not sensitize mice to *M. bovis* purified protein derivative.

Reintroduction of *Mycobacterium bovis* into livestock via infected imported animals and wildlife reservoirs is one of the primary reasons why *M. bovis* has not been eradicated from livestock. Although control measures such as traditional “test-and-slaughter” approaches have been somewhat successful in the United States, these programs are limited to the identification of animals already infected with *M. bovis*. Similar programs have not been successful in eliminating the risk of *M. bovis* infections in livestock via wildlife reservoirs present in countries outside the United States. Vaccination of healthy cattle against *M. bovis* would prevent their infection by disease, imported animals and wildlife reservoirs. Such a vaccination program would be most effective in areas where *M. bovis* is endemic (e.g., Ireland and New Zealand) and test-and-slaughter control measures are impractical.

Bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*, has been used for immunoprophylaxis against human tuberculosis for most of the 20th century (30). Buddle and colleagues (6, 7) recently addressed the potential of BCG to protect cattle against virulent *M. bovis*. Although the majority of vaccinated animals did not develop lesions following intratracheal immunization with BCG and challenge with virulent *M. bovis*, every vaccinated animal tested positive for *M. bovis* by different diagnostic tests, the skin test and the whole-blood gamma interferon (IFN-γ) test. Data from these experiments demonstrated that BCG would not be an ideal vaccine in cattle since the use of this vaccine eliminated the only available diagnostic tests for the detection of *M. bovis* in cattle.

Initial studies by Hubbard and colleagues (14) followed by those by Pal and Horowitz (26) demonstrated the potential of *Mycobacterium tuberculosis* culture filtrate proteins (CFP) to protect animals against challenge with *M. tuberculosis*. Later experiments showed that CFP in a mild adjuvant (dimethyldioctadecyl ammonium bromide [DDA]) could enhance this protection (3). Roberts and colleagues confirmed Andersen’s results and also made the important observation that CFP-based vaccines did not appear to sensitize mice to commercial *M. tuberculosis* purified protein derivative (PPD) (28).

To date no one has addressed the potential of *M. bovis* CFP as an effective vaccine against bovine tuberculosis. We have previously demonstrated that *M. bovis* CFP was capable of inducing the secretion of the protective cytokine IFN-γ from *M. bovis*-sensitized cells (unpublished data) similar to responses seen in animal models of *M. tuberculosis*. This suggested that *M. bovis* CFP may be capable of eliciting protective immune responses in vaccinated animals. Based on these observations we hypothesized that *M. bovis* CFP-based vaccines would protect animals against a sublethal challenge of virulent *M. bovis* without sensitizing vaccinated animals to PPD.

The CFP used in these experiments were secreted by the virulent *M. bovis* field isolates 85-4036, 86-579, and 90-3053 (a kind gift from R. Ellis, Colorado State University, Fort Collins) and purified as previously described for *M. tuberculosis* (28). Briefly, early- and mid-log-phase cultures of each *M. bovis* strain were filter sterilized and concentrated via ultrafiltration over an Amicon ultrafiltration stirred cell (Amicon, Danvers, Mass.) fitted with a PM 10 membrane (Millipore, Bedford, Mass.). Samples were further concentrated to a final volume of about 5 ml by centrifugation in a Savant Speed-Vac (Savant Instruments, Holbrook, N.Y.). The protein content of each sample was assessed by the commercial bicinchoninic acid protein assay (Pierce, Rockford, Ill.) performed according to the manufacturer’s instructions; these preparations were aliquoted in 1-ml samples and stored at −20°C.

Equal amounts of *M. bovis* CFP from each isolate were pooled and tested with three different adjuvants. The concentration of CFP (100 μg/injection) was chosen based on dose-response curves generated by Andersen in his study of *M. tuberculosis* CFP as a vaccine against *M. tuberculosis* infections (3). The three adjuvants chosen were DDA, Imject Alum (aluminum hydroxide), and Quil-A.

DDA was prepared as previously described (3). Briefly, a 2.5-mg/ml suspension in sterile, pyrogen-free water (Sigma, St. Louis, Mo.) was heated at 80°C for 10 min to achieve a homogeneous suspension. After cooling to room temperature, DDA was diluted 1:10 with CFP resuspended in sterile, pyrogen-free phosphate-buffered saline (PBS) (Sigma). Each vaccine consisted of 50 μg of DDA plus 100 μg of CFP per 200-μl injection. Imject Alum (Pierce) was diluted 1:2 with CFP in sterile, pyrogen-free PBS (Sigma) according to the manufacturer’s instructions. The resulting vaccines consisted of 2.2 mg of Imject Alum plus 100 μg of CFP per 200-μl injection. Quil-A (Accurate, Westbury, N.Y.) was resuspended at 200 μg/ml in sterile, pyrogen-free PBS (Sigma) and diluted 1:2 with CFP resuspended in sterile, pyrogen-free PBS (Sigma). The resulting vaccine consisted of 20 μg of Quil-A plus 100 μg of CFP per 200-μl injection. This concentration was recommended as appropriate for mice by C. Kensil (Cambridge Biotech Corporation, Worcester, Mass.), who has worked extensively with...
Quil-A to determine its potency as an adjuvant in a number of vaccine models (8, 17, 18, 20, 25, 29).

A murine model of *M. bovis* infections was used in this study. Six- to eight-week-old female, specific-pathogen-free C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in a biohazard level 3 safety facility for the duration of the experiment. Mice were given food and water ad libitum.

Mice were immunized with 200 μl of each experimental CFP subunit vaccine subcutaneously, at two time points 3 weeks apart. A single dose (10⁶ bacteria/200 μl) of *M. bovis* BCG Pasteur (Mycobacterial Research Laboratories, Colorado State University, Fort Collins) was injected subcutaneously into control mice when the subunit vaccine groups received their second immunization. The dose of BCG administered in these experiments was routinely used by the Mycobacterial Research Laboratories to protect mice against a sublethal aerosol challenge of virulent *M. tuberculosis*. Negative controls consisted of mice injected subcutaneously with 200 μl of sterile, pyrogen-free saline (Sigma) or 2.2 mg of Imject Alum (Pierce) per 200 μl. There were five mice per experimental group.

Low-dose aerosol infections were performed as previously described (23). Briefly, mice were placed in a Middlebrook Airborne Infection Apparatus (Glas-Col, Terre Haute, Ind.). The nebulizer compartment was filled with a 10-ml suspension of *M. bovis* 86-579 at 10⁶ bacteria/ml diluted in sterile water delivering 50 to 100 bacilli into the lungs during a 40-min exposure time.

Three days before challenge mice were tested for sensitivity to commercial *M. bovis* PPD (National Veterinary Services Laboratory, Ames, Iowa). The protein content of PPD was determined by the bicinchoninic acid protein assay kit (Pierce). PPD was diluted in sterile, pyrogen-free PBS (Sigma) to 200 μg/ml. Fifty microliters (10 μg) of this solution was injected into the right hind footpad of each mouse. As a negative control bovine serum albumin (BSA, 10 μg/50 μl) (Sigma) was prepared in sterile, pyrogen-free PBS (Sigma) and injected into the contralateral footpad. Forty-eight hours later delayed-type hypersensitivity (DTH) responses were assessed as differences in footpad thickness between test and negative-control footpads. There were no measurable indurations 24 h after injection of PPD (data not shown).

Mice were killed 30 days postinfection by asphyxiation with CO₂. Lungs and spleens were aseptically removed and homogenized in sterile saline with a straight-sided, fixed-motor-drive homogenizer (Glas-Col). Bacterial loads of each organ were enumerated by plating 10-fold serial dilutions of organ homogenates on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.). Colonies were counted approximately 3 weeks after plating. In a separate experiment unvaccinated mice were exposed to low-dose aerosols of *M. bovis* 86-579 and killed 0, 20, 40, and 60 days postinfection. Bacteria were enumerated from the lungs as described above.

The significances of footpad indurations between groups of mice which received subunit vaccines, alum, or BCG and mice which received saline were measured by one-way analysis of variance followed by Tukey-Kramer’s multiple-comparisons test using Graphpad InStat (Graphpad Software, San Diego, Calif.). Efficacies of different vaccine preparations were compared by Student’s *t* test by assuming equal variance with Microsoft Excel 4.0 for Macintosh (Microsoft Corporation, Seattle, Wash.).

To establish virulence of *M. bovis* 86-579 in mice, bacteria from lungs of infected mice were enumerated at predetermined time points. *M. bovis* 86-579 followed growth patterns similar to those noted for *M. tuberculosis* (22) (Fig. 1). The bacteria grew logarithmically for the first 20 days followed by a period in which the infection entered a chronic phase. Notably, *M. bovis* 86-579 grew to nearly 10⁷ CFU/lung within the first 20 days, unlike the virulent laboratory strain *M. tuberculosis* Erdman (22). Mice maintained this number of bacteria in the lungs throughout the course of the experiment, i.e., 60 days. This pattern of growth was similar to those noted for clinical isolates of *M. tuberculosis* (26) and suggested increased virulence of *M. bovis* field isolates over that of laboratory strains of *M. tuberculosis*.

After we established that *M. bovis* 86-579 could grow and sustain infection in mice, we determined if *M. bovis* CFP-based subunit vaccines would induce sensitivity to PPD. Mice vaccinated with either subunit vaccines or *M. bovis* BCG Pasteur were analyzed for DTH responses in their footpads following injections of commercial *M. bovis* PPD or the irrelevant antigen BSA. Of the three subunit vaccines, CFP plus DDA and CFP plus Quil-A failed to significantly sensitize mice to *M. bovis* PPD (*P* > 0.05) (Fig. 2). In contrast, both BCG and alum plus CFP significantly sensitized mice to *M. bovis* PPD (*P* < 0.05).

To determine if subunit vaccines consisting of *M. bovis* CFP in adjuvant could protect mice against an aerosol challenge with virulent *M. bovis*, three different adjuvants were individually mixed with CFP, and the responses were compared to those in mice immunized with saline (negative control) or BCG (positive control). The number of bacteria retrieved from lungs of mice following an aerosol challenge and the levels of protection afforded by each vaccine preparation are listed in Table 1. Each CFP vaccine, regardless of adjuvant, was able to significantly enhance protective immune responses in vaccinated animals (*P* < 0.02). Mice vaccinated with CFP in the mild adjuvant DDA exhibited the strongest protection against an aerosol challenge with virulent *M. bovis*, with almost 10³ fewer bacteria per lung than saline controls. In fact, CFP in DDA elicited stronger protective responses than BCG, in that CFP in DDA resulted in approximately 1 log fewer organisms in the lungs than vaccination with BCG.

In this study we have shown that, in a murine model, CFP in a variety of adjuvants were capable of inducing a protective immune response in vaccinated animals against an aerosol challenge of *M. bovis*.
challenged with *M. bovis*. Importantly, we have also demonstrated that mice immunized with highly purified CFP of *M. bovis* mixed in either DDA or Quil-A adjuvants were not sensitized to commercial *M. bovis* PPD. Among the subunit vaccines tested, CFP in the mild adjuvant DDA induced the greatest protective immune response. These exceeded responses engendered by *M. bovis* BCG, currently the only vaccine tested in cattle to protect against *M. bovis* infections. CFP in DDA was also superior to BCG in that it did not sensitize animals to PPD, thus preserving the skin test as an important diagnostic tool.

The hypothesis that, when used as a vaccine, CFP would not sensitize animals is controversial since components of culture filtrate from mycobacteria have been tested as skin test antigens (11, 13). The center of this controversy revolved around the hypothesis that DTH responses to PPD and protection against mycobacterial infections were dissociable events. It has been commonly believed that the ability of an animal to mount a cutaneous DTH response against PPD was a direct indicator of protective immunity; however, in the past few years evidence from several laboratories suggested that this relationship was not as simplistic as previously believed. Cloned “DTH effector” cells have been demonstrated to transfer DTH reactivity to PPD but not protection against a subsequent challenge with *M. tuberculosis* (15). Others have shown that vaccination with heat-killed *M. bovis* BCG sensitized animals to PPD but did not enhance protective antimycobacterial immune responses (2, 27). Recent experiments using a variety of gene-disrupted mice have shown that mice lacking IFN-γ could not control *M. tuberculosis* infections but exhibited aggressive DTH responses against PPD (9), while mice lacking intracellular adhesion molecules (type I) control mycobacterial growth but do not mount DTH responses to PPD (16). Together these observations support the hypothesis that DTH and protective immune responses, which occur during mycobacterial infections are dissociable events. The data presented in our study support this hypothesis, in that mice vaccinated with alum plus CFP generated DTH responses against PPD yet exhibited the weakest protective immune responses against an aerosol challenge with *M. bovis*.

Additionally, experiments performed by Anacker and colleagues (2) and Orme (23) suggest that DTH and protective immune responses may have been dominated by different sets of proteins. For example, the DTH responses may have been optimally elicited by proteins which are abundant in PPD, i.e., heat shock proteins, other stress proteins, and cell wall-associated proteins (12). These antigens were found in negligible amounts, if at all, in the CFP preparations used in the study described here (5). This is not to say that CFP could not elicit DTH responses in infected animals. Previous studies in our laboratory have shown that CFP was a highly specific skin test antigen for the identification of cattle naturally infected with *M. bovis* (5). However, it is also important to note that in those experiments CFP preparations were far less sensitive than PPD (i.e., less able to identify infected animals), which suggested that the number of DTH effector cells primed against CFP were far fewer than those primed against PPD antigens. Alternatively, the mechanism by which the proteins are delivered to the host may influence the type or strength of the response generated against the antigens. The data presented here supports this observation in that CFP mixed in the appropriate adjuvant were unable to significantly sensitize animals to *M. bovis* PPD. One explanation for this phenomenon of protection without sensitization is the different mechanisms of antigen delivery by different adjuvants. For example, the mechanism by which alum works for the delivery of antigens to the host is very different from that of either Quil-A or DDA (1, 21). These varying mechanisms of antigen delivery may explain why mice vaccinated with alum plus CFP generated large inductions in response to PPD, whereas mice vaccinated with the same proteins in different adjuvants did not.

The study we have presented here complements similar observations by Andersen (3), who used *M. tuberculosis* CFP in DDA to protect against intravenous challenges of virulent *M. tuberculosis*. Andersen’s observations were further investigated by Linblad et al., who studied the effects of cytokines as coadjuvants in subunit vaccine preparations (19). In these experiments the addition of interleukin 2 (IL-2) to CFP increased the concentration of IFN-γ secreted from T cells in immunized mice, while the addition of IL-12 enhanced protective responses originally observed with CFP in adjuvant alone. Data from our own laboratory suggests that addition of IL-2 to CFP-based vaccines optimizes protective immune responses.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Burden (CFU/lung)</th>
<th>Resistance (10^5 CFU/lung)</th>
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<tbody>
<tr>
<td>DDA + CFP</td>
<td>(1.2 ± 0.23) × 10^6</td>
<td>8.33 ± 0.19</td>
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<tr>
<td>BCG</td>
<td>(1.0 ± 0.16) × 10^6</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>Quil-A + CFP</td>
<td>(1.6 ± 0.24) × 10^6</td>
<td>0.10 ± 0.20</td>
</tr>
<tr>
<td>Alum + CFP</td>
<td>(1.6 ± 0.16) × 10^6</td>
<td>0.06 ± 0.15</td>
</tr>
<tr>
<td>Alum</td>
<td>(3.0 ± 0.20) × 10^6</td>
<td>0.33 ± 0.19</td>
</tr>
<tr>
<td>Saline</td>
<td>(3.0 ± 0.05) × 10^7</td>
<td>NA *</td>
</tr>
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* Mice were sacrificed 30 days after challenge.

* Values are means ± standard errors of the means (five mice per group).

* Significantly different (P ≤ 0.05) from saline control value as calculated by Student’s t test.

* NA, not applicable.
against an aerosol challenge with *M. tuberculosis* in vaccinated mice (4). It is therefore reasonable to suggest that addition of IL-2 to our CFP-plus-DDA vaccine would further enhance resistance against an aerosol challenge of virulent *M. bovis*. These studies are currently under way in our laboratory.

The results of this study demonstrate that, in a murine model, *M. bovis* CFP may be an effective vaccine against virulent *M. bovis* infections. We have also shown that CFP in the mild adjuvant DDA was more effective at stimulating protective immune responses against *M. bovis* than *M. bovis* BCG, currently the only vaccine tested in cattle. We also made the important observation that CFP vaccines in either DDA or Quil-A do not significantly sensitize animals to PPD, thus preserving the only diagnostic test currently available for the detection of *M. bovis* infections in cattle. An additional significant observation was that this lack of sensitization to PPD was dependent upon the adjuvant used for vaccination of animals. Development of a vaccine against *M. bovis* for use in cattle would eliminate the danger of reintroducing the disease and facilitate the eradication process, primarily in areas where the disease is endemic and the elimination of wildlife reservoirs is impractical. The data presented here suggests that CFP in appropriate adjuvants may function as effective vaccines against *M. bovis* without compromising the reliability of the diagnostic tests for bovine tuberculosis. We are currently testing the efficacy of CFP-based vaccines in the natural host, cattle.

This work was supported by the USDA Graduate Fellowship program (92-38420-7330) and the Colorado State University College Research Council.

**REFERENCES**


