Subcutaneous Administration of a 10-Fold-Lower Dose of a Commercial Human Tuberculosis Vaccine, *Mycobacterium bovis* Bacillus Calmette-Guérin Danish, Induced Levels of Protection against Bovine Tuberculosis and Responses in the Tuberculin Intradermal Test Similar to Those Induced by a Standard Cattle Dose

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Vaccination of cattle with a commercial human tuberculosis (TB) vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Danish, at a dose equivalent to 5 human doses of BCG has protected these animals against TB in field and experimental trials. There is interest in determining whether a 10-fold-lower dose could still protect cattle but not induce a tuberculin intradermal test response. Two groups of calves (9/group) were vaccinated subcutaneously with a lyophilized BCG Danish vaccine containing either 0.5 (1 × 10^5 to 4 × 10^6 CFU) or 5 (1 × 10^7 to 4 × 10^8 CFU) human doses of BCG Danish, with an additional group of 10 calves serving as nonvaccinated controls. Fifteen weeks after vaccination, these animals were challenged intratracheally with 5 × 10^4 CFU of virulent *M. bovis* and another 15 weeks later were slaughtered and examined for the presence of tuberculous lesions. Vaccination of the calves with either 0.5 or 5 equivalent human doses of BCG Danish induced similar levels of protection against challenge with *M. bovis*, with both groups showing significant reductions in the pathological and microbiological parameters compared to those for the control group (P < 0.05). Vaccination with either of the two BCG doses induced similar numbers of animals responding to the tuberculin intradermal test at 11 weeks postvaccination. Vaccination with a 0.5 equivalent human dose of a commercial lyophilized BCG vaccine can protect cattle against challenge with *M. bovis*.

The human tuberculosis (TB) vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), has been shown to induce a significant level of protection in cattle against bovine TB, caused by *M. bovis*, in experimental challenge and field trials (1). However, the vaccine has not been used in national bovine TB control programs because cattle, at least in the first year after vaccination, may react positively to the tuberculin intradermal test, and protection may not be complete (2). Recent research showed that the problem of BCG vaccination compromising the conventional bovine TB diagnostic tests can be overcome by using tests differentiating infected from vaccinated animals (DIVA). For these tests, antigens from the DIVA reservoirs of *M. bovis* are not affordable or acceptable.

Commercially produced lyophilized BCG Danish vaccine (Statens Serum Institute, Denmark) at a dose equivalent to 5 human doses (1 × 10^6 to 4 × 10^8 CFU) administered subcutaneously to cattle has been shown to induce protection against TB equivalent to that with freshly prepared cultures of BCG Pasteur and Danish strains of BCG (7). This provides a potential licensed product for use in cattle. A series of recent studies confirmed that this dose of the commercial BCG vaccine induced a significant level of protection in cattle against experimental challenge and natural exposure to *Mycobacterium bovis* (8, 9, 10). Information on the effectiveness of a lower dose of this vaccine would be valuable to determine whether small variations within the normal range in the dose of different batches of the commercially prepared vaccine affected efficacy and to provide assurance that the specified target dose is well above the minimal effective titer. In addition, the cost of the vaccine can potentially be reduced if a lower dose is efficacious. The aim of the current study was to determine whether a 10-fold-lower dose of the commercial BCG Danish vaccine administered subcutaneously induces protection against bovine TB and responses in the standard tuberculin intradermal tests equivalent to those of cattle vaccinated at the higher dose.

MATERIALS AND METHODS

Animals. Twenty-eight female Friesian cross calves, 5 to 6 months old, were obtained from a herd which was accredited as TB free for the previous 10 years and was from an area of New Zealand where both farmed and
feral animals were free of TB. Prior to the studies, the cattle tested negative for bovine TB in whole-blood IFN-γ tests. The cattle grazed on pasture in a biacontaminated unit. All animal procedures were approved by an independent animal ethics committee.

**Bacterial strains and vaccines.** The lyophilized *M. bovis* BCG Danish 1331 vaccine (Statens Serum Institute, Copenhagen, Denmark) formulated for human use was used to vaccinate the calves. For the high dose of vaccine, the vial of vaccine was reconstituted in 1 ml Sauton’s medium (Statens Serum Institute), and the 0.3-ml dose contained 1 × 10⁶ to 4 × 10⁶ CFU as specified by the manufacturer and was used within the specified shelf life. The low dose of vaccine was prepared by reconstituting the vial of vaccine in 2 ml of Sauton’s medium, and the 0.1-ml dose contained 1 × 10⁵ to 4 × 10⁵ CFU of BCG. *M. bovis* strain WAG202, originally isolated from a tuberculous possum in New Zealand, was used as the challenge strain and had been used in previous vaccination/challenge studies in cattle (7,11). The bacteria were grown to mid-log phase in Tween-albumin broth (Dubos broth base; Difco Laboratories, Detroit, MI) supplemented with 0.006% (vol/vol) alkalined oleic acid, 0.5% (wt/vol) albumin fraction V, and 0.25% (wt/vol) glucose. Dilutions were made in Tween-albumin broth to obtain the dose for inoculation. The numbers of CFU inoculated were determined retrospectively by plating 10-fold dilutions of the tissues on Middlebrook 7H11 (Difco) supplemented with 0.5% (wt/vol) albumin, 0.2% (wt/vol) glucose, and 1% (wt/vol) sodium pyruvate.

**Vaccination.** The calves were divided into two groups of 9 calves and one group of 10 calves using a randomized stratified sampling system so that all groups contained animals with a similar distribution of IFN-γ responses to the avian PPD in the weeks prior to the start of the study. Nine calves in one group were each injected subcutaneously in the left side of the neck with 5 human doses of BCG Danish, 9 calves in a second group were each injected in a similar manner with 0.5 human dose equivalents of BCG Danish, and an additional 10 calves served as nonvaccinated (negative) controls.

**M. bovis challenge and necropsy procedure.** The calves were challenged intratracheally with 5 × 10⁵ CFU of virulent *M. bovis* as previously described (11) at 15 weeks after vaccination. All cattle were killed 15 weeks after challenge. The procedures for identifying macroscopic tuberculous lesions and processing for histopathology were described previously (11). A lung lesion score was calculated by counting the total number of lesions and applying a score as follows: 0, no lesions; 1, 1 to 9 lesions; 2, 10 to 29 lesions; 3, 30 to 99 lesions; 4, 100 to 199 lesions; and 5, ≥200 lesions. A total lymph node lesion score per animal was calculated by pooling the scores for four pulmonary lymph nodes (left and right bronchial and anterior and posterior mediastinal). The scores for individual lymph nodes were 0 for no lesions, 1 for 1 to 19 small lesions (1 to 3 mm in diameter), 2 for 20 small or medium-sized lesions (4 to 6 mm in diameter), and 3 for large lesions (>6 mm in diameter). Samples from four pulmonary lymph nodes were collected from all of the animals for bacterial cultures and histology analyses to confirm *M. bovis* infection. Additional samples were collected from any tuberculous-like lesions observed in the lungs or other lymph nodes or organs. For bacterial cultures, the tissue samples were homogenized in a Ten Broeck grinder (Wheaton, Millville, NJ), decontaminated in 0.75% cetylpyridinium chloride for 1 h, centrifuged at 3,500 × g for 20 min (included in the decontamination time), and processed for the isolation of mycobacteria as described previously (11).

**IFN-γ assay.** Heparinized blood samples were collected from the calves at regular intervals to analyze their cellular immune responses. The blood samples (1.5 ml) were dispersed into wells of a 24-well plate, and preservative-free bovine PPD or avian PPD prepared from Mycobacterium avium (24 μg/ml final concentrations) (Prionics, Schlieren-Zurich, Switzerland) or phosphate-buffered saline (negative control) was added. The blood cultures were set up within 6 h following the blood collection. After incubation at 37°C for 24 h, the plasma supernatants were harvested, and their IFN-γ levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Prionics). The results are expressed as ng/ml of IFN-γ using a standard curve.

**Tuberculin intradermal test.** The comparative cervical tuberculin intradermal test was undertaken at 11 weeks postvaccination and 13 weeks postchallenge. For this test, the cattle were inoculated intradermally with 0.1-ml volumes containing either 2,500 IU of avian PPD or 5,000 IU of bovine PPD (AsureQuality, Upper Hutt, New Zealand) at separate sites on the right side of the neck. The skin fold thicknesses were measured with calipers prior to and 72 h after injection of the PPDs.

**Statistical analyses.** The statistical analyses for IFN-γ responses and intraderrmal test data were undertaken using R 2.15 software (12). A mixed-effects model was applied to the natural log-transformed IFN-γ responses; time, group, and their interaction were fixed effects, and animal was treated as a random effect. A two-way ANOVA was applied to the natural log-transformed IFN-γ levels for each group and the treatment effects. Post hoc comparisons were made using the Tukey’s HSD test. Statistical significance was denoted when *P* was <0.05.

### TABLE 1 Summary of pathological and bacteriological findings following *Mycobacterium bovis* challenge

<table>
<thead>
<tr>
<th>BCG vaccine group</th>
<th>Proportions with lesions in:</th>
<th>Median LN lesion score (range)</th>
<th>Median lung lesion score (range)</th>
<th>No. of lesioned LNs/animal (mean ± SEM)</th>
<th>No. of <em>M. bovis</em> culture-positive LNs/animal (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High dose</td>
<td>LN: 7/9, Lung: 3/9</td>
<td>2 (0–5)</td>
<td>0.0003</td>
<td>1.2 ± 0.3</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Low dose</td>
<td>LN: 5/9, Lung: 2/9</td>
<td>1 (0–4)</td>
<td>0.0005</td>
<td>1.3 ± 0.5</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Nonvaccinated</td>
<td>10/10</td>
<td>4.5 (3–5)</td>
<td></td>
<td>3.3 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

a LN, lymph node.

b Scores for individual nodes: 0, no lesions; 1, 1 to 19 small lesions (1 to 3 mm in diameter); 2, ≥20 small or medium-size lesions (4 to 6 mm in diameter); 3, large lesions (>6 mm in diameter). The total lesion scores for four LNs were pooled.

c Lung lesion scores: 0, no lesions; 1, 1 to 9 lesions; 2, 10 to 29 lesions; 3, 30 to 99 lesions; 4, 100 to 199 lesions; 5, ≥200 lesions.

d Significantly different from the nonvaccinated group (*P* < 0.05).
and mean numbers of M. bovis culture-positive lymph nodes/animal than those for the nonvaccinated group (pairwise comparison of each BCG-vaccinated group with the nonvaccinated group, \( P < 0.05 \)). Only the low-dose BCG group had a significantly lower proportion of animals with lymph node lesions than that for the nonvaccinated group \( (P < 0.05) \). However, there were no statistically significant differences in the disease parameters between the two BCG-vaccinated groups. The lesions were typical of those for bovine TB, with multiple small (1- to 3-mm-diameter) calcified lesions in the lung and variable-sized calcified lesions in the pulmonary lymph nodes (1 to 20 mm in diameter). No macroscopic tuberculous lesions were observed outside the pulmonary cavity.

These findings confirmed results from an earlier study in which similar levels of protection against TB were induced when cattle were vaccinated with freshly prepared cultures of BCG Pasteur at doses of \( 6 \times 10^5 \) and \( 6 \times 10^6 \) CFU of BCG and challenged with 800 CFU of M. bovis \( (11) \). Further support for the observation of low-dose BCG imparting protection was provided by studies in deer where subcutaneous vaccination with \( 5 \times 10^4 \) and \( 5 \times 10^6 \) CFU of BCG Pasteur induced comparable levels of protection against infection and disease following intratonsillar challenge with 200 to 500 CFU of M. bovis \( (13) \). Interestingly, vaccination with a higher dose of \( 5 \times 10^6 \) CFU of BCG in that study did not induce protection against infection and evoked immune responses with a bias toward type 2 rather than type 1 reactivity.

A cervical tuberculin intradermal test can be either a single intradermal injection of bovine PPD in the midcervical region or a comparative cervical intradermal test where bovine and avian PPDs are injected intradermally at adjacent sites in the midcervical region. Results for the current study are shown for the bovine PPD response alone and for the bovine PPD minus the avian PPD response \( (B - A) \) response. The single bovine PPD test is used in continental Europe, and an increase in swelling after 72 h is classified as a positive result \( (14) \). The comparative cervical intradermal test is used in areas such as the United Kingdom or Ireland, where cattle are frequently exposed to organisms of the M. avium complex or to environmental mycobacteria. Increases in skin fold swellings at the two sites are compared 72 h after injection, and in the United Kingdom, the standard interpretation for a positive reaction corresponds to an increase of \( > 4 \) mm in the skin fold thickness at the bovine PPD site compared with that for the avian PPD site. The implementation of a more stringent interpretation of the test or retesting of animals which have shown an inconclusive reaction (comparative increase of 1 to 4 mm) has been introduced in different situations \( (14) \). In the current study, the mean intradermal test responses to bovine PPD and bovine PPD minus avian PPD \( (B - A) \) responses at 11 weeks postvaccination were similar for the two BCG-vaccinated groups, and the mean responses for these groups were significantly greater than those for the nonvaccinated group \( (P < 0.05) \) (Table 2). At 11 weeks postvaccination, the numbers of animals in the high-BCG-dose group which had responses of \( \geq 1 \) mm for bovine PPD and \( \geq 4 \) mm for bovine PPD and \( B - A \) were 8, 6, and 2, respectively, while the equivalent numbers of animals in the low-BCG-dose group were 8, 3, and 3, respectively. No responses were observed in the nonvaccinated control group at this time. Findings from the current study indicate that vaccination with doses of \( 10^5 \) to \( 10^6 \) CFU of BCG was associated with false-positive results in the single bovine PPD and comparative intradermal tests. Tests to differentiate the infected from the vaccinated animals would be required, but such tests were not performed in this study. However, a rapid decline in the intradermal test sensitivity has been observed by 6 months after BCG vaccination; hence, it is advisable to delay the tuberculin intradermal testing until at least 6 months following the BCG vaccination. The positive responses in the comparative cervical intradermal tests were reduced from 80% to 8% between 6 and 9 months postvaccination \( (2) \). The costs for conducting these differentiating tests can be minimized by retesting only those animals which were positive in the tuberculin intradermal test.

The results of the intradermal testing at 13 weeks postchallenge showed that all animals in the BCG-vaccinated and nonvaccinated groups had increases in skin fold thicknesses of \( \geq 4 \) mm for bovine PPD and for \( B - A \). However, the mean responses for the high- and low-BCG-dose groups for bovine PPD and the mean response for the low-BCG-dose group for avian PPD were significantly lower than those for the nonvaccinated group \( (P < 0.05) \). Inter-

![FIG 1](image-url) Total lesion scores (lung and pulmonary lymph nodes) following challenge for the high-dose-BCG, low-dose-BCG, and nonvaccinated groups. Medians are indicated by horizontal lines. ***, Significantly different from the nonvaccinated group \( (P < 0.001) \).

| TABLE 2 | Intradermal test responses postvaccination and postchallenge for groups of cattle vaccinated with a high dose \( (1 \times 10^6 \) to \( 4 \times 10^6 \) CFU) of BCG or nonvaccinated cattle |
|----------|---------------------------------------------------|--------------------------------------------------|
|          | Skin test response to PPD (mean ± SEM) (mm) at*: | 13 wk postchallenge |
|          | 11 wk postvaccination | 13 wk postchallenge |
|          | Bovine PPD | Avian PPD | B - A | Bovine PPD | Avian PPD | B - A |
| BCG vaccination group |          |          | |          |          | |
| High dose   | 4.1 ± 0.7\textsuperscript{a} | 1.4 ± 0.5\textsuperscript{a} | 2.7 ± 0.5\textsuperscript{a} | 16.4 ± 1.8\textsuperscript{b} | 3.1 ± 0.8 | 14.0 ± 1.3 |
| Low dose    | 3.6 ± 1.0\textsuperscript{a} | 0.7 ± 0.4 | 2.9 ± 0.8\textsuperscript{a} | 15.5 ± 1.6\textsuperscript{b} | 2.4 ± 0.8\textsuperscript{b} | 13.1 ± 1.4 |
| Nonvaccinated | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 22.5 ± 1.7 | 5.5 ± 0.8 | 17.1 ± 1.3 |

\textsuperscript{a}Increases in skin fold thickness between 0 and 72 h after injection.

\textsuperscript{b}Significantly different from the nonvaccinated group \( (P < 0.05) \).
The responses to the avian PPD were also lower in the vaccinated groups. Consequently, no significant differences between the vaccinated and control groups were observed when the comparative B – A responses were tabulated (Table 2).

Vaccination with the high or low dose of BCG resulted in the release of IFN-γ in the bovine PPD-stimulated blood cultures. The kinetics of the responses for bovine PPD for the two BCG groups were similar, with no significant differences between these two groups (Fig. 2). Comparisons between the three groups showed that the two BCG groups had significantly greater mean IFN-γ responses to the bovine PPD (natural log-transformed data) at 3, 6, 8, and 11 weeks postvaccination and at 15 weeks postvaccination for the high-dose group than those for the nonvaccinated group (P < 0.05). The IFN-γ test is increasingly being used as an ancillary test for the diagnosis of bovine TB in cattle, and a common cutoff used in this test is the bovine PPD response in optical density (OD) units minus the avian PPD response of 0.100 OD (15). At 15 weeks postvaccination, the numbers of IFN-γ responses were tabulated (Table 2). The kinetics of the responses for bovine PPD for the two BCG groups were similar, with no significant differences between these two groups (Fig. 2). Comparisons between the three groups showed that the two BCG groups had significantly greater mean IFN-γ responses to the bovine PPD (natural log-transformed data) at 3, 6, 8, and 11 weeks postvaccination and at 15 weeks postvaccination for the high-dose group than those for the nonvaccinated group (P < 0.05). The IFN-γ test is increasingly being used as an ancillary test for the diagnosis of bovine TB in cattle, and a common cutoff used in this test is the bovine PPD response in optical density (OD) units minus the avian PPD response of 0.100 OD (15). At 15 weeks postvaccination, the numbers of IFN-γ responses were tabulated (Table 2).

In conclusion, a lower dose of the commercial lyophilized BCG Danish vaccine, equivalent to a 0.5 human dose, was as effective as a 10-fold-higher dose and can be used in a vaccination program for the control of bovine TB. However, both doses of BCG induced false-positive responses in the tuberculin intradermal tests, and tests to differentiate infected from vaccinated animals would be required.

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