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

Bryce M. Buddle,a R. Glyn Hewinson,b H. Martin Vordermeier,b D. Neil Wedlocka

AgResearch, Hopkirk Research Institute, Palmerston North, New Zealand; Animal Health and Veterinary Laboratories Agency, Weybridge, Surrey, United Kingdom

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 (n = 9/group) were vaccinated subcutaneously with a lyophilized BCG Danish vaccine containing either 0.5 (1 × 10^5 to 4 × 10^5 CFU) or 5 (1 × 10^6 to 4 × 10^6 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 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 *Mycobacterium tuberculosis* complex which are not expressed by BCG are used instead of purified protein derivative (PPD) prepared from *M. bovis* (bovine PPD) in the intradermal test (3) or in the whole-blood gamma interferon (IFN-γ) assay (4, 5). Furthermore, complete protection against TB by vaccination may not be essential for reducing the prevalence of TB in cattle. BCG vaccination of humans against TB is considered a valuable control strategy even though BCG vaccination of newborns and infants significantly reduces the risk of tuberculosis by only 50%, on average (per a meta-analysis of studies [6]). Renewed interest in the use of TB vaccines for cattle has arisen from the realization of the financial impact of bovine TB on animal health and trade and the fact that “test and slaughter” bovine TB control programs have been less effective in countries with wildlife reservoirs of *M. bovis* infection and those where these programs 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 × 10^6 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 biocontainment 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 Institutue, 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 0.5-ml dose contained 1×10^6 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 1×10^5 to 4×10^6 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) alkalized 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 onto 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 5×10^5 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 PPV 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 (AurexQuality, 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 intradermal 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 and challenge (an indicator variable for identifying the period [before or after challenge]) were random effects. The intradermal test data were analyzed using the t test. Fisher’s exact test was used for comparing the proportion of animals with lung or lymph node lesions. For the remaining data, the statistical analyses were undertaken using Minitab 15. The lesion scores were compared using the pairwise Mann-Whitney U test. The mean numbers of lesioned lymph nodes/animal or nodes culture positive for *M. bovis* were analyzed using analysis of variance (ANOVA) with Tukey’s multiple comparisons. Statistical significance was denoted when *P* was <0.05.

**RESULTS AND DISCUSSION**

The subcutaneous vaccination of the calves with the high (5 human doses) or the low (0.5 human dose) dose of reconstituted lyophilized BCG Danish induced significant protection against the experimental challenge with *M. bovis* compared to that of the nonvaccinated group (Table 1 and Fig. 1). The two BCG-vaccinated groups had significantly lower proportions of animals with lung lesions, lower pulmonary lymph node, lung, and total lesion scores, and lower mean numbers of lesioned lymph nodes/animal

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

| BCG vaccine group | Proportions with lesions in: | Median lung lesion score (range) | No. of lesioned LNs/animal (mean ± SEM) |
|-------------------|-----------------------------|----------------------------------|----------------------------------------|
| High dose         | LN², Lung                   | 0 (0–5)⁴                       | 1.2 ± 0.3⁴                              |
| Low dose          |                             | 0 (0–4)⁴                       | 1.3 ± 0.5⁴                              |
| Nonvaccinated     |                             | 4.5 (3–5)                      | 3.3 ± 0.2                              |

²LN, lymph node.

* Statistical analyses. The statistical analyses for IFN-γ responses and intradermal 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 and challenge (an indicator variable for identifying the period [before or after challenge]) were random effects. The intradermal test data were analyzed using the t test. Fisher’s exact test was used for comparing the proportion of animals with lung or lymph node lesions. For the remaining data, the statistical analyses were undertaken using Minitab 15. The lesion scores were compared using the pairwise Mann-Whitney U test. The mean numbers of lesioned lymph nodes/animal or nodes culture positive for *M. bovis* were analyzed using analysis of variance (ANOVA) with Tukey’s multiple comparisons. Statistical significance was denoted when *P* was <0.05.

**RESULTS AND DISCUSSION**

The subcutaneous vaccination of the calves with the high (5 human doses) or the low (0.5 human dose) dose of reconstituted lyophilized BCG Danish induced significant protection against the experimental challenge with *M. bovis* compared to that of the nonvaccinated group (Table 1 and Fig. 1). The two BCG-vaccinated groups had significantly lower proportions of animals with lung lesions, lower pulmonary lymph node, lung, and total lesion scores, and lower mean numbers of lesioned lymph nodes/animal
FIG 1 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).

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 × 10^5 and 6 × 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 × 10^4 and 5 × 10^5 CFU of BCG Pasteur induced comparable levels of protection against infection and disease following intratarsillar challenge with 200 to 500 CFU of *M. bovis* (13). Interestingly, vaccination with a higher dose of 5 × 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 thicknesses 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 ≥1 mm for bovine PPD and ≥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 ≥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-

| BCG vaccination group | Skin test response to PPD (mean ± SEM) (mm) at a/ | 13 wk postchallenge |
|-----------------------|-----------------------------------------------|---------------------|
|                       | 11 wk postvaccination | 13 wk postchallenge |
|                       | Bovine PPD | Avian PPD | B − A | Bovine PPD | Avian PPD | B − A |
| High dose             | 4.1 ± 0.7b | 1.4 ± 0.5a | 2.7 ± 0.5a | 16.4 ± 1.8a | 3.1 ± 0.8 | 14.0 ± 1.3 |
| Low dose              | 3.6 ± 1.0b | 0.7 ± 0.4a | 2.9 ± 0.8a | 15.5 ± 1.6a | 2.4 ± 0.8b | 13.1 ± 1.4 |
| Nonvaccinated         | 0.0 ± 0.0a | 0.0 ± 0.0a | 0.0 ± 0.0a | 22.5 ± 1.7a | 5.5 ± 0.8b | 17.1 ± 1.3 |

**a** Increases in skin fold thickness between 0 and 72 h after injection.

**b** Significantly different from the nonvaccinated group (P < 0.05).
 resendly, 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 animals which had positive IFN-γ responses above the cutoff were 5, 4, and 0 for the high-BCG-dose, low-BCG-dose, and nonvaccinated groups, respectively. Following the challenge, there was a rapid increase in the IFN-γ responses to bovine PPD for all groups and from 5 weeks postchallenge, the mean IFN-γ response to bovine PPD was greater for the nonvaccinated group, although not significantly compared to those for the BCG-vaccinated groups (data not shown).

In the New Zealand situation, an efficacious TB vaccine could be used to protect cattle herds within areas under long-term maintenance control where the costs of possum control are high relative to the number of herds at risk. These areas would not receive possum control, relying on vaccinations to keep TB rates low in herds. Costs for possum control can be saved by not undertaking control in these areas where cattle are vaccinated, allowing possum control funding to be diverted to areas where TB could be eradicated from the possum populations. DIVA tests, either intradermal tests (3) or whole-blood IFN-γ tests (4, 5) using mycobacterial antigens absent from BCG, would be used for retesting any tuberculin intradermal test-positive animals.

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.

**REFERENCES**

1. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. 2012. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine 30:2611–2622.
2. Whelan AO, Coad M, Upadhyay BL, Clifford DJ, Hewinson RG, Vordermeier HM. 2011. Lack of correlation between BCG-induced skin test reactivity and protective immunity in cattle. Vaccine 29:5453–5458.
3. Whelan AO, Clifford D, Upadhyay B, Breadon EL, McNair J, Hewinson RG, Vordermeier HM. 2010. Development of a skin test for bovine tuberculosis for differentiating infected from vaccinated animals. J. Clin. Microbiol. 48:3176–3181.
4. Buddle BM, Parlane NA, Keen DL, Aldwell FE, Pollock JM, Lighthoby K, Andersen P. 1999. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. Clin. Diag. Lab. Immunol. 6:1–5.
5. Vordermeier M, Gordon SV, Hewinson RG. 2011. *Mycobacterium bovis* antigens for the differential diagnosis of vaccinated and infected cattle. Vet. Microbiol. 151:8–13.
6. Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick D, Fineberg HV. 1995. The efficacy of bacillus Calmette-Guérin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. Pediatrics 96:29–35.
7. Wedlock DN, Denis M, Vordermeier HM, Hewinson RG, Buddle BM. 2007. Vaccination of cattle with Danish and Pasteur strains of *Mycobacterium bovis* BCG induce different levels of IFN-γ vaccination, but induce similar levels of protection against bovine tuberculosis. Vet. Immunol. Immunopathol. 118:50–58.
8. Vordermeier HM, Villarreal-Ramos B, Cockle PJ, McAulay M, Rhodes SG, Thacker T, Gilbert SC, McShane H, Hill AVS, Xing Z, Hewinson RG. 2009. Viral booster vaccines improve *Mycobacterium bovis* BCG-induced protection against bovine tuberculosis. Infect. Immun. 77:3364–3373.
9. Amenu GG, Vordermeier HM, Aseffa AA, Young DB, Hewinson RG. 2010. Field evaluation of the efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin against bovine tuberculosis in neonatal calves. Clin. Vaccine Immunol. 17:1533–1538.
10. Thom ML, McAulay M, Vordermeier HM, Clifford D, Hewinson RG, Villarreal-Ramos B, Hope JC. 2012. Duration of immunity against *Mycobacterium bovis* following neonatal vaccination with bacillus Calmette-Guérin Danish: significant protection against infection at 12, but not 24, months. Clin. Vaccine Immunol. 19:1254–1260.
11. Buddle BM, de Lisle GW, Pfeffer A, Aldwell FE. 1995. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. Vaccine 13:1123–1130.
12. Pinheiro JD, Bates S, DeRoy Sarkar D, R Development Core Team. 2013. nlme: linear and nonlinear mixed effects models. R package version 3.1-107. R Foundation for Statistical Computing, Vienna, Austria.
13. Griffith JFT, Mackintosh CG, Slobbe L, Thomson AJ, Buchan GS. 1999. Vaccine protocols to optimise the protective efficacy of BCG. Tuber. Lung Dis. 79:135–143.
14. Monaghan ML, Doherty ML, Collins JD, Kasda JF, Quinn PJ. 1994. The tuberculin test. Vet. Microbiol. 40:111–124.
15. Buddle BM, Livingstone PG, de Lisle GW. 2009. Advances in ante mortem diagnosis of tuberculosis in cattle. N. Z. Vet. J. 57:173–180.