The Order of Prime-Boost Vaccination of Neonatal Calves with *Mycobacterium bovis* BCG and a DNA Vaccine Encoding Mycobacterial Proteins Hsp65, Hsp70, and Apa Is Not Critical for Enhancing Protection against Bovine Tuberculosis


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Priming neonatal calves at birth with a *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine and boosting with a DNA vaccine consisting of plasmids encoding mycobacterial antigens Hsp65, Hsp70, and Apa or the reverse prime-boost sequence induced similar levels of protection against experimental challenge with *Mycobacterium bovis*. When *M. bovis* was isolated from a thoracic lymph node following challenge, the two groups of calves given the prime-boost regimen had significantly lower numbers of *M. bovis* isolates than those vaccinated with BCG alone. These observations suggest that the exact sequence of administration of a prime-boost vaccination regimen in a neonatal animal model is not critical to the development of immunity.

Tuberculosis is a major health problem in many areas of the world, and control of the disease in both humans and cattle hinges on the development of an improved vaccine or vaccination strategy. The only vaccine currently available is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Meta-analysis of human BCG vaccination trials has revealed a reduction in the risk of tuberculosis following BCG vaccination and a strong association between early-life BCG vaccination and reduced risks of meningeal and miliary tuberculosis in children (4). However, BCG vaccination does not induce complete protection against pulmonary tuberculosis in adults, and it is important to develop improved vaccination strategies.

An alternative vaccination strategy would be to use a prime-boost regimen that combines BCG (as either the prime or the boost) with a novel candidate vaccine. Recent studies on the enhancement of the effectiveness of BCG, using mice and cattle, have shown that priming with a DNA vaccine and boosting with BCG induced a higher level of protection than BCG alone (5, 6, 14). For human infants, it would be most practical to vaccinate with BCG at birth and later boost with another vaccine, as there are acknowledged health benefits from vaccinating infants with BCG at birth. Calves are useful models for testing efficacies of vaccines for human tuberculosis, as the disease has a similar pathogenesis and neonatal BCG vaccination induces a high level of protection against tuberculous lesions (3). The aim of the present study was to determine whether priming with BCG and boosting with a DNA vaccine consisting of plasmids encoding mycobacterial antigens Hsp65, Hsp70, and Apa induced protection against an experimental challenge with *Mycobacterium bovis*. Equivalent to the protection induced with a DNA prime/BCG boost combination. Secondly, we sought to determine whether these vaccination strategies were more efficacious than BCG alone in newborn calves.

Friesian-cross calves were removed from their mothers 4 to 12 h after birth and taken to a calf-rearing facility. The cows were from a tuberculosis-free accredited herd from an area of New Zealand where both farmed animals and wildlife were free of tuberculosis. The calves were moved to a high-security containment unit at 17 weeks of age. Animal ethics approval was granted for all animal manipulations.

The plasmids pCMV4.65, pCMV4.70, and pCMV4.apa, encoding mycobacterial antigens Hsp65, Hsp70, and Apa, respectively, were constructed as described previously (8, 14). The *M. bovis* BCG strain Pasteur 11732P2 was used as the vaccine strain, and *M. bovis* WAg202, originally isolated from a tuberculous possum in New Zealand, was used as the challenge strain. These strains had been used in previous vaccination/challenge studies in cattle (1, 3, 14) and were grown to mid-log phase as described previously (1).

When the calves were 24 to 48 h of age, they were randomly divided into four groups. Animals in group 1 (n = 11) were vaccinated with BCG at 24 to 48 h of age and boosted with the DNA vaccine at 6 and 9 weeks of age (BCG/DNA); animals in group 2 (n = 10) were vaccinated with the DNA vaccine at 24 to 48 h of age and at 3 weeks of age and were boosted with BCG at 6 weeks of age (DNA/BCG); animals in group 3 (n = 10) were vaccinated with BCG at 24 to 48 h of age and at 3 weeks of age and were boosted with BCG at 6 weeks of age (BCG/BCG); and animals in group 4 (n = 10) were vaccinated with BCG at 24 to 48 h of age and at 3 weeks of age and were boosted with DNA at 6 weeks of age (BCG/DNA/DNA).
were vaccinated with BCG at 24 to 48 h of age (BCG alone); and group 4 animals (n = 11) were not vaccinated (nonvaccinated). For BCG vaccination, 10^6 CFU of BCG was given subcutaneously in the neck. For DNA vaccination, a total of 0.5 mg of each DNA plasmid was given as a split dose, 0.35 mg intramuscularly and 0.15 mg intradermally. One calf from group 2 died of unknown causes at 19 days of age and was excluded from the analysis.

The calves were challenged intratracheally at 17 to 18 weeks of age with 5 × 10^3 CFU of virulent M. bovis as described previously (1). All cattle were killed and necropsied 16 weeks after challenge. Procedures for identifying macroscopic tuberculous lesions and processing for bacterial culture and histology have been described previously (1). Samples from four thoracic lymph nodes (left and right bronchial and anterior and posterior mediastinal) were collected from all of the animals for bacterial culture and histology. Additional samples were collected from observed tuberculous lesions in lungs and other lymph nodes or organs.

After the vaccinations, gamma interferon (IFN-γ) and interleukin 2 (IL-2) responses were measured in plasma supernatants from whole blood cultures incubated at 37°C for 24 h with purified protein derivative from M. bovis (bovine PPD) (final concentration, 24 μg/ml; CSL Ltd., Parkville, Australia) or phosphate-buffered saline (3). IFN-γ levels in the plasma supernatants were measured by using a sandwich enzyme-linked immunosorbent assay kit (CSL Ltd.) as described previously (13, 15). IL-2 levels were assayed by a bioassay as described previously (15), based on the uptake of [3H]thymidine by concanavalin A-stimulated lymphoblasts. Results were expressed as stimulation indices, defined as mean cpm for bovine PPD plasma supernatant/mean cpm for the phosphate-buffered saline plasma supernatant. Delayed hypersensitivity (DTH) to bovine PPD was measured 13 weeks after the first vaccination and 15 weeks after the M. bovis challenge (1 week prior to slaughter). Animals were inoculated intradermally with a 0.1-ml volume containing 100 μg bovine PPD in the side of the neck and the results expressed as the differences in skin thickness between the time of inoculation and 72 h later. Analyses of IFN-γ and IL-2 responses were undertaken by using analyses of variance on log_{10} transformed data, while DTH responses were analyzed by using raw data. The log_{10} transformed bacterial counts were analyzed by using the Kruskal-Wallis test and the proportion of animals with lesions by using Fisher’s exact test.

All three vaccinated groups exhibited significant reductions in four pathological parameters and two microbiological parameters of protection compared to the nonvaccinated group (P < 0.05) (Table 1). Reduced pathological parameters included the proportion of animals with lesions in the lung or lymph nodes and the mean number of thoracic lymph nodes per animal with macroscopic or microscopic lesions. The microbiological parameters that were reduced were the mean number of thoracic lymph nodes per animal that were M. bovis culture positive and the median bacterial count for these lymph nodes. Although there were no significant differences between individual vaccinated groups, the majority of the pathological and microbiological findings associated with the lymph nodes were lower for the groups vaccinated with a combination of BCG and DNA vaccines than for those vaccinated with BCG alone. Furthermore, when M. bovis was isolated from a thoracic lymph node, the median bacterial count (25th and 75th percentiles) for these nodes (1.440 [1.041 and 1.949] log_{10} CFU/g of tissue; n = 31) was significantly lower than that for the group vaccinated with BCG alone (1.849 [1.452 and 2.808] log_{10} CFU/g of tissue; n = 20) (P < 0.05). As our previous studies had shown that mycobacteria were very rarely isolated from nonlesioned sites in the lungs of experimentally infected cattle (B. Buddle, unpublished data), samples of lung were collected only for bacterial culture from lesions. Due to the small number of animals from the vaccinated groups with lesions in their lungs, bacterial counts from lung lesions were analyzed by comparing the mean count for the vaccinated groups combined with that for the nonvaccinated group. The median bacterial count (25th and 75th percentiles) for the lung lesions of the vaccinated groups combined (2.655 [2.433 and 3.321] log_{10} CFU/g of tissue) was significantly less than that for the nonvaccinated group (3.198 [2.964 and 4.412] log_{10} CFU/g of tissue) (P < 0.05).

All of the macroscopic tuberculous lesions were confined to the thoracic cavity. Lesions in the lung consisted of small nodules 2 to 5 mm in diameter with yellow, caseous centers, and lesions in lymph nodes were 1 to 15 mm in diameter with yellow, calcified and/or caseous centers. In addition to the three vaccinated groups having a lower proportion of animals with tuberculous lesions, for those vaccinated animals with
lesions, the numbers and sizes of the lesions were reduced compared to the numbers and sizes of observed lesions in the nonvaccinated animals. The proportion of nonvaccinated calves with more than 100 lung lesions/animal was 9/11, compared to only 2/10 of the vaccinated animals that had lesions in their lungs (P < 0.05). Similarly, the majority of macroscopic lesions in lymph nodes of the nonvaccinated group were ≥5 mm in diameter (22 of the 32 lesioned lymph nodes), while there were no lesions that were ≥5 mm in diameter in vaccinated animals, from a total of 10 lesioned lymph nodes. Histologically, the tuberculous granulomata in lesions in the lung and lymph nodes in the nonvaccinated animals had a central necrotic area which was usually mineralized and surrounded by a wide band of granulomatous tissue. The granulomatous tissue contained epithelioid macrophages, giant cells, and lymphocytes, as well as small numbers of neutrophils, and was walled off by fibrous tissue. When lung lesions were present in vaccinated animals, they were similar to the observed lesions in the nonvaccinated animals, while the majority of observed lesions in the lymph nodes of the vaccinated animals, particularly for the BCG/DNA group, appeared to be earlier in development, as they comprised only granulomatous tissue with no necrosis or fibrous encapsulation.

Vaccination with the DNA vaccine containing plasmids encoding Hsp65, Hsp70, and Apa has been shown to induce protection in mice (7). When mice were primed with this vaccine and boosted with BCG, immunity was further enhanced and the vaccine was shown to induce protection against tuberculosis that was significantly greater than that seen with BCG alone (6). In contrast, when this DNA vaccine was used with cattle, no protection could be demonstrated when the vaccine was used alone, while the combination of DNA prime/BCG boost enhanced protection compared to BCG alone (14). The current study differs from the earlier study with calves, as the first vaccination was administered at 24 to 48 h of birth, compared to 5 to 6 months of age. In developing countries, human infants are vaccinated within hours of birth, and hence, the findings from the current study are more relevant to the human situation. The finding that it made no difference whether the DNA vaccine was used as a prime or boost for the BCG vaccine is important, as priming with BCG at birth would be the preferred vaccination sequence for human infants. When M. bovis was isolated from a thoracic lymph node following challenge, the two groups of calves given prime-boost regimens had significantly lower numbers of M. bovis isolated than those vaccinated with BCG alone, indicating an enhanced protection with these combinations. This result is very encouraging, since vaccination with BCG alone at birth induces a very high level of protection against tuberculosis for calves compared to BCG vaccination at 5 to 6 months of age (2, 3). Although earlier studies with mice promoted the use of a DNA vaccine as a prime, a recent report has shown that a BCG prime and a DNA vaccine boost provided superior protection against tuberculosis compared to vaccination with BCG alone (11), which concurs with results from the current study.

The monitoring of IFN-γ or IL-2 responses to bovine PPD from whole blood cultures following vaccination showed that the BCG/DNA and the BCG-alone groups had significantly higher IFN-γ and IL-2 responses at 3, 6, 9, and 14 weeks than the nonvaccinated group (P < 0.05) (Fig. 1). Interestingly, boosting with the DNA vaccine (BCG/DNA group) resulted in a significant enhancement of IL-2 responses at 12 weeks of age compared to treatment with BCG alone (P < 0.05), while there was no boosting of IFN-γ responses. Priming with the DNA vaccine did not induce IFN-γ or IL-2 responses to bovine PPD at 3 or 6 weeks of age, while boosting with the BCG vaccine significantly enhanced responses compared to nonvaccination conditions (P < 0.05). The nonvaccinated calves produced an IFN-γ response to bovine PPD, peaking at approximately 9 weeks of age, which is similar to outcomes seen in previous studies with neonatal calves (3, 12). This immune response did not affect the development of protective immunity against tuberculosis when calves were vaccinated with BCG at 6 weeks of age (3). Many uninfected calves less than 3 months of age can produce an IFN-γ response to specific antigens of the Mycobacterium tuberculosis complex, such as ESAT-6 and CFP10.
sequent boosting with a DNA vaccine. That the efficacy of BCG vaccination can be improved by sub-
treated to newborn infants, and our findings provide evidence.
comes when administered as a prime-boost regimen (9, 10). It
pointing results, while DNA vaccines produced better out-
trials. In humans, DNA vaccines alone have provided disap-
prime/DNA boost could find applications in human clinical
hanced the efficacy of BCG. These findings suggest that a BCG
a DNA prime/BCG boost and that these combinations en-

doing mycobacterial proteins Hsp65, Hsp70, and Apa pro-
tions that were significantly greater than those for the non-
week challenge (P < 0.05) (data not shown).

Our results showed that priming neonatal animals with BCG
results, while DNA vaccines produced better out-

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