Comparison of the Protective Efficacy of Bacille Calmette-Guérin Vaccination against Aerosol Challenge with *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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The aim of this study was to compare protection by bacille Calmette-Guérin (BCG) vaccination against aerosol challenge with either *Mycobacterium bovis* or *Mycobacterium tuberculosis* in guinea pigs. Animals were challenged 5 weeks after vaccination with 10 or 100 lung lesion–forming units (lfu) of *M. bovis* or *M. tuberculosis*. Four weeks after challenge, numbers of lung lesions and counts of viable mycobacteria in spleens were high in saline-immunized animals. In contrast, BCG vaccination resulted in fewer lung lesions; after challenge with 10 and 100 lfu, the reduction was greater in animals infected with *M. bovis* (mean number of lesions, 1.17 and 31.2, respectively; *P < .02*) than in those infected with *M. tuberculosis* (mean number of lesions, 16.2 and 75.8, respectively). No mycobacteria were recovered from spleens of BCG-vaccinated animals after challenge with 10 lfu of *M. bovis*, whereas 4 of 6 animals had detectable spleen mycobacterial counts after challenge with *M. tuberculosis*. Collectively, the results suggest that BCG vaccination may confer greater protection against challenge with *M. bovis* than challenge with *M. tuberculosis*.

The development of tuberculosis (TB) vaccines is driven by both human and animal health requirements. There is global recognition of the need to develop an improved vaccine to provide greater protection against TB in humans, and in Great Britain, a recent independent scientific review for the government concluded that the best prospect for control of TB in the British herd is the development of a vaccine to protect cattle against TB [1]. The existing vaccine against TB (BCG vaccine) is an attenuated strain of *Mycobacterium bovis* and is currently widely administered as part of the World Health Organization Expanded Programme for Immunization. A series of clinical trials have shown that BCG vaccine protects against primary childhood infection with *Mycobacterium tuberculosis* but affords variable protection against the predominantly pulmonary form of the disease in adults [2–4]. The variable protective efficacy of BCG vaccine has also been observed against *M. bovis* infection in cattle [5]. Genetic analysis of BCG has revealed that loss of virulence is associated with large genetic lesions from within a number of regions of the *M. bovis* genome [6, 7]. This finding has led to the suggestion that one approach to the development of improved TB vaccines is to generate attenuated strains by specifically inactivating genes required for intracellular survival and virulence of *M. tuberculosis* and *M. bovis* [8].

At present, however, there is little evidence to suggest whether attenuated strains of *M. bovis* or *M. tuberculosis* are preferable as vaccine candidates against aerosol exposure to the homologous species. The aim of this study, therefore, was to compare the ability of BCG vaccine to protect guinea pigs against aerosol challenge with *M. bovis* and *M. tuberculosis*. Protection was assessed by both the extent of pulmonary disease and the degree of bacterial dissemination as measured by counts of viable mycobacteria in spleens.

**Methods**

Bacterial strains and media. Lyophilized *M. bovis* BCG Pasteur strain (Statens Serum Institut, Copenhagen) was cultured in 10 mL of M-ADC-TW broth [9] for 7 days and stored at −80°C in seed lots. Seed lots of *M. tuberculosis* H37Rv, (National Collection of Type Cultures 7416) were grown on Middlebrook 7H10 agar (Difco, Detroit) containing 0.2% (vol/vol) glycerol and 10% (vol/vol) OADC enrichment, harvested, and stored at −70°C as a dense suspension in deionized water. The *M. bovis* strain used in this study (*M. bovis* 1692/96) was isolated from a cow reactive to tuberculin testing in 1996 and cultured at Veterinary Laboratories Agency (Weybridge, UK), as described below. For enumeration, *M. tuberculosis* H37Rv and the *M. bovis* BCG Pasteur strain were plated on Middlebrook 7H10 agar containing 0.2% (vol/vol) glycerol and 10% (vol/vol) OADC enrichment. *M. bovis* 1692/96 was plated on Middlebrook 7H10 agar containing 4.16 mg of sodium.
pyruvate/mL and 10% (vol/vol) OADC enrichment. Where necessary, serial dilution of bacterial suspensions was done in water containing 0.05% (vol/vol) polysorbate 80 to maintain dispersion.

**Vaccination.** Groups of 6 female Dunkin-Hartley guinea pigs (weight range, 200–250 g) were immunized sc in the nape on a single occasion with either 250 μL of saline or 5 × 10⁶ cfu of the *M. bovis* BCG Pasteur strain in an identical volume of saline.

**Aerosol generation and challenge.** Mycobacterial aerosols were generated by a Collison 3-jet nebulizer in conjunction with a contained Henderson apparatus [10]. The mean relative humidity ± SD was controlled at 65% ± 3% and the airflow rate at 55 L/min, to ensure reproducible infective doses. In preliminary experiments, fine-particle aerosols (mean particle diameter, 2 μm; range, 0.5–7.0 μm) generated from suspensions of ~6.0 and 7.0 log₁₀ cfu/mL were delivered for 5 min directly to the snout of each animal and resulted in an average of 10 and 100 lesions, respectively, on the surface of the lung 4 weeks after challenge (data not shown). It is assumed that 1 lung lesion is formed from 1 cfu, but in this low-dose model, an accurate assessment of cfu is not possible; therefore, the term “lung lesion–forming unit” (lfu) is used instead. In this study, animals were challenged with predicted retained inhaled doses of 10 or 100 lfu of *M. bovis* or *M. tuberculosis* 5 weeks after BCG vaccination in a blind, randomized study design.

**Vaccine efficacy.** Animals were monitored regularly for weight and temperature changes as indicators of distress. Protection was assessed by the extent of pulmonary disease and by the degree of dissemination to the spleen. Four weeks after infection, the animals were killed. The spleens were removed aseptically and were homogenized in 5 mL of sterile distilled water, and the lungs were fixed in formal-buffered saline. The number of lung lesions (>1 mm in diameter) were counted on the dorsal surface of the lung. Counts of viable mycobacteria were measured on the homogenized spleen samples by decimal dilution of homogenates and plating aliquots on Middlebrook 7H10 agar, as described above. Counts of viable mycobacteria were log-transformed, and the mean count of viable organisms was expressed per milliliter of homogenized spleen. The detection limit was 5 cfu/mL of homogenized sample. Data were compared by use of the unpaired *t* test; *P* < .05 was considered significant.

**Results**

In animals immunized with saline, a challenge dose of 10 lfu of *M. bovis* or *M. tuberculosis* resulted in mean lung lesion counts of 41.8 and 41.3, respectively (table 1), and mean spleen mycobacterial counts of 4.26 and 4.78 log₁₀ cfu/mL, respectively (figure 1). A challenge dose of 100 lfu of *M. bovis* and of *M. tuberculosis* resulted in more lung lesions (139.7 and 187.4, respectively; table 1), whereas the mean spleen mycobacterial counts were 5.01 and 5.64 log₁₀ cfu, respectively (figure 1). Numbers of lung lesions (*P* < .01) and spleen mycobacterial counts (*P* < .05) after challenge with 100 lfu were significantly higher than those after challenge with 10 lfu of either species.

Numbers of lung lesions in vaccinated animals are shown in table 1. BCG-vaccinated animals had fewer lung lesions than did saline-immunized animals under all challenge conditions. However, the extent of reduction varied depending on the challenge species used in the model. At a challenge dose of 10 lfu, BCG vaccine significantly reduced the mean number of lung lesions (± SD) to 1.2 ± 1.2 lesions after challenge with *M. bovis*, compared with 16.2 ± 12.3 lesions after challenge with *M. tubercu-

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**Table 1.** Efficacy of BCG vaccination against challenge with 2 doses of *Mycobacterium bovis* 1692/96 and *Mycobacterium tuberculosis* H₃₇R₇, in female Dunkin-Hartley guinea pigs.

<table>
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<tr>
<th>Animal</th>
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Mean ± SD 41.8 ± 25.3 41.3 ± 9.0 1.17 ± 1.1 16.2 ± 11.2 139.7 ± 59.6 187.4 ± 32.1 31.2 ± 17.4 75.8 ± 44.7

**NOTE.** Data are no. of lung lesions (>1 mm in diameter) on the dorsal surface of the lung. lfu, lung lesion–forming unit.

*Animal excluded from the study since all lobes were covered with confluent foci 2–3 mm in diameter.*
The greater protection afforded by BCG vaccination against challenge with *M. bovis* compared with challenge with *M. tuberculosis* was statistically significant (*P < .02*). At a challenge dose of 100 lfu, a similar trend was observed, but the difference in protection did not reach statistical significance (*P = .06*).

The mycobacterial counts detected in spleens support the above-mentioned trend. In BCG-vaccinated animals, no mycobacteria could be detected in macerated spleens from any guinea pig after challenge with 10 lfu of *M. bovis* (minimum detection level, 5 cfu/mL). Similarly, mycobacteria were not detected in the spleens of 4 of 6 guinea pigs challenged with 100 lfu of *M. bovis* (figure 1). In contrast, aerosol challenge with 10 and 100 lfu of *M. tuberculosis* resulted in mean spleen mycobacterial counts of 0.86 and 1.94 log$_{10}$ cfu/mL, respectively (figure 1). Mycobacteria could not be detected in the spleens of 2 guinea pigs at each challenge dose.

The differences in spleen mycobacterial counts in vaccinated animals challenged with *M. bovis* and *M. tuberculosis* was statistically significant at 10 lfu (*P < .05*) but not at 100 lfu. However, because enrichment cultures of the spleen samples were not performed, it is possible that some mycobacteria were present in those samples in which no viable counts were measured (minimum detection level, 5 cfu/mL). If a positive value is used to replace the zero counts in the statistical analyses, the significance of the difference between the spleen mycobacterial counts after challenge with 10 lfu ranges from *P < .05* to *P = .06*, depending on the value used (i.e., 1.25–2.5 cfu).

Discussion

To mimic natural infection as closely as possible, guinea pig aerosol challenge models of both *M. tuberculosis* and *M. bovis* infection were established with reliable and reproducible delivery of retained inhaled doses as low as 10 lfu in the lung. The term “lung lesion-forming unit” was used because preliminary experiments showed that the total retained inhaled dose of delivered mycobacteria was at, or lower than, the detection limit for determining cfu in homogenized lung specimens obtained at the time of challenge. Key features of the aerosol challenge were the ability to deliver small infectious particles of defined size (mean particle diameter, 2 μm) directly to the snout of individual animals that resulted in the penetration of low numbers of mycobacteria deep into the alveoli in a dose-dependent manner. Challenge with 100 lfu of *M. tuberculosis H$_3$R$_{v}$ or *M. bovis* 1692/96 resulted in significantly higher numbers of lung lesions (*P < .01*) and spleen mycobacterial counts (*P < .05*) than did challenge with 10 lfu in saline-immunized animals.

BCG vaccination conferred greater protection, in terms of numbers of lung lesions and spleen mycobacterial counts, against challenge with *M. bovis* than against challenge with *M. tuberculosis*. However, the extent of this reduction was dependent on the challenge dose. Significantly fewer lung lesions were present after challenge with *M. bovis* at the lower dose (10 lfu; *P < .02*); the difference at 100 lfu approached significance (*P = .06*). Similarly, no mycobacteria were detected in the spleens of any of the animals exposed to low-dose (10 lfu) aerosol challenge with *M. bovis*, whereas 4 of 6 guinea pigs had detectable mycobacterial counts following challenge with *M. tuberculosis*.

A number of strategies have been proposed for the development of candidates for an improved TB vaccine for use in either humans or animals. These strategies include the use of protein subunit and DNA vaccines, as well as the evaluation of randomly or rationally attenuated mycobacterial strains [8]. With respect to the latter approach, there have been no studies to ascertain whether strains of *M. bovis* or *M. tuberculosis* may have inherent advantages as candidates for the development of attenuated strains as vaccines. Although only 1 challenge strain of each species was compared, the data presented in this pilot study on pulmonary lesions and spleen mycobacterial counts suggest that there may be advantages in using representatives of the homologous species for the development of attenuated strains as vaccines against either *M. tuberculosis* or *M. bovis*. Further studies that use a wider range of challenge strains would be needed to confirm this paradigm.

References