Tuberculosis (TB) is one of the most prevalent causes of death from infectious diseases in the world. As is the case for many intracellular pathogens, cell-mediated immunity plays an important role in host protection against TB (25, 29). In particular, gamma interferon (IFN-γ)-secreting T cells have been shown to be important for the protective immune response (17). The only vaccine currently available against TB is the attenuated Mycobacterium bovis strain bacillus Calmette-Guérin (BCG). The efficacy of this vaccine varies from 0 to 80% in different populations, with a consistently low efficacy in many tropical regions of the world where the vaccine is most needed (15, 16, 35, 38). The reason for the failure of BCG in some populations has been a subject of debate since the 1950s, and many different hypotheses have been suggested to explain the observed variation. Some investigators have suggested that differences in the strain of BCG (23), the age at vaccination (40), or methodological differences are important factors for the variation reported (8). The most widely accepted hypothesis relates the efficacy of BCG to geographic location, with low to nondetectable levels of protection against pulmonary TB seen in tropical regions such as Africa and India, where exposure to nontuberculous mycobacteria is common (15). One exception from this general rule is the consistent high efficacy when BCG is used to vaccinate newborns. Neonatal vaccination with BCG imparts protection against the childhood manifestations of TB (in particular, meningitis) (1, 9, 24), but the efficacy wanes over a period of 10 to 15 years, and therefore it does not prevent against the later breakdown with pulmonary TB in the adult population in the third world (37).

There is convincing evidence that exposure of laboratory animals to environmental mycobacteria can provide some protection against infection with Mycobacterium tuberculosis (7, 14, 20, 30, 33). The influence of such cross protection on the efficacy of subsequent BCG vaccination is not yet clarified, but based on animal experiments, it has been suggested that the protection provided by environmental mycobacteria may partly mask the effect of a subsequent BCG vaccination (33, 42) or that environmental mycobacteria have a direct antagonistic influence on subsequent BCG vaccination (34, 36). Our study demonstrates that prior sensitization with environmental mycobacteria can inhibit BCG multiplication and thereby prevent the induction of an efficient BCG-mediated immune response and protection against TB challenge. Interestingly, different species isolated from soil and sputum in Karong, Malawi, an area in which BCG vaccination has been shown to provide no protection against TB (22), differed in their ability to inhibit BCG multiplication. In contrast, a TB subunit vaccine had the same protective effect in naive and sensitized animals.

MATERIALS AND METHODS

Animals. These studies were performed with pathogen-free 6- to 12-week-old CBA/J and C57BL/6J female mice, purchased from Bomholtgaard, Ry, Denmark, or, in some of the experiments, purchased from Harlan UK, Ltd., Belton, England, or Harlan Interfauna Ibérica, Barcelona, Spain.

Bacteria. Mycobacterium avium (ATCC 15769), Mycobacterium scrofulaceum (ATCC 19275), and Mycobacterium vaccae (ATCC 15483) were grown in 7H9 broth until the mid-log phase of the bacterial growth. Mycobacterium tuberculosis (Edman) was grown at 37°C on Löwenstein-Jensen medium or in suspension in modified Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose. In prepa-
A crude BCG antigen preparation (BCG Ag) was produced as an ammonium sulfate-precipitated culture filtrate from cultures at week 6 as described in reference 2. In one of the experiments (see Fig. 4), the BCG responses to an ammonium sulfate-precipitated extract of the cell wall were measured as described elsewhere (31). These two preparations were found to give similar responses in vitro.

**TABLE 1.** Sensitization with environmental mycobacteria blocks the protective effect of BCG

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Spleen</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log$_{10}$ resistance$^a$</td>
<td>CFU</td>
</tr>
<tr>
<td>Naive</td>
<td>4.44 ± 0.13</td>
<td>6.34 ± 0.11</td>
</tr>
<tr>
<td>BCG</td>
<td>3.76 ± 0.16</td>
<td>5.21 ± 0.08</td>
</tr>
<tr>
<td>Sensitization</td>
<td>4.36 ± 0.17</td>
<td>6.14 ± 0.11</td>
</tr>
<tr>
<td>Sensitization + BCG</td>
<td>4.33 ± 0.17</td>
<td>6.25 ± 0.06</td>
</tr>
</tbody>
</table>

$^a$ Naive or sensitized mice were BCG vaccinated (5 × 10$^4$ CFU) followed by aerosol challenge with virulent M. tuberculosis.

$^b$ The experiment was repeated twice with similar results.

$^c$ Bacterial numbers determined by growth of individual whole-organ homogenates 6 weeks postinfection.

$^d$ Protective effect expressed as the log$_{10}$ reduction in bacterial loads compared to those of naive mice. Bacterial numbers significantly different (P < 0.05) from those seen in naive mice are indicated by an asterisk.

**RESULTS**

The multiplication of BCG is inhibited in mice sensitized with certain environmental mycobacteria. We inoculated CBA/J mice s.c. three times at 2-week intervals with a mixture

<table>
<thead>
<tr>
<th>Vaccine group$^a$</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log$_{10}$ CFU$^a$</td>
<td>Log$_{10}$ resistance$^a$</td>
</tr>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>6.36 ± 0.08</td>
<td>4.71 ± 0.05</td>
</tr>
<tr>
<td>BCG</td>
<td>5.83 ± 0.06</td>
<td>0.39*</td>
</tr>
<tr>
<td>DDA-MPL</td>
<td>6.34 ± 0.09</td>
<td>4.94 ± 0.12</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>5.76 ± 0.09</td>
<td>0.60*</td>
</tr>
<tr>
<td>Sensitized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.18 ± 0.08</td>
<td>4.82 ± 0.16</td>
</tr>
<tr>
<td>BCG</td>
<td>6.27 ± 0.07</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DDA-MPL</td>
<td>6.39 ± 0.05</td>
<td>4.73 ± 0.11</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>6.54 ± 0.16</td>
<td>0.44*</td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive</td>
<td>6.88 ± 0.12</td>
<td>5.10 ± 0.18</td>
</tr>
<tr>
<td>DDA-MPL</td>
<td>7.19 ± 0.05</td>
<td>5.48 ± 0.11</td>
</tr>
<tr>
<td>Ag85B–ESAT-6</td>
<td>6.03 ± 0.12</td>
<td>0.85*</td>
</tr>
<tr>
<td>Sensitized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.30 ± 0.08</td>
<td>4.39 ± 0.09</td>
</tr>
<tr>
<td>DDA-MPL</td>
<td>6.49 ± 0.05</td>
<td>4.27 ± 0.08</td>
</tr>
<tr>
<td>Ag85B–ESAT-6</td>
<td>5.37 ± 0.14</td>
<td>0.93*</td>
</tr>
</tbody>
</table>

$^a$ Naive or sensitized mice were immunized s.c. with BCG or injected three times with a subunit vaccine emulsified in DDA-MPL.

$^b$ Bacterial numbers are given as log$_{10}$ CFU of M. tuberculosis isolated from the lung and spleen 6 weeks after aerosol challenge with virulent M. tuberculosis.

$^c$ Protective effects of the two vaccines are expressed as log$_{10}$ reductions in bacterial numbers compared to those in unvaccinated control mice. Bacterial numbers significantly different from those seen in control mice are indicated by an asterisk.

**TABLE 2.** Bacterial numbers in organs of naive and sensitized mice after vaccination and aerosol challenge with virulent M. tuberculosis
of the mycobacterial strains *M. avium*, *M. scrofulaceum*, and *M. vaccae*. These species have repeatedly been isolated from soil and water samples in tropical regions (21). Three weeks postinoculation, a low but significant mycobacterium-specific recall response was measured in the spleen, with detectable levels of IFN-γ release in response to BCG Ag. (1.26 ± 0.01 ng/ml) (data not shown). The BCG Ag preparation gave no IFN-γ release (<0.05 ng/ml) from splenocytes isolated from naive mice. No IL-4 or IL-5 was detected in any of the supernatants. Three weeks after the last inoculation with environmental mycobacteria, we subjected the mice to 4 weeks of chemotherapy to clear remaining live mycobacteria. After the end of chemotherapy treatment, no environmental mycobacteria were detected in any of the target organs (liver, spleen, and lymph nodes).

We inoculated groups of sensitized and age-matched naive CBA/J mice i.v. 1 week after the end of chemotherapy treatment with 5 × 10⁶ BCG and monitored the growth in the spleen and liver over time. Sensitization with environmental mycobacteria resulted in inhibition of the initial multiplication of BCG in the spleen and liver (Fig. 1A). In naive mice, the initial multiplication of BCG resulted in 10- to 30-fold more bacteria in the spleen postinoculation than in sensitized mice. A difference was also seen after a conventional s.c. vaccination, although the bacterial numbers were at lower levels (data not shown). Similar data were obtained with C57BL/6J mice, which are more susceptible to BCG (11). In this strain, larger differences in BCG numbers were found between sensitized and nonsensitized mice (Fig. 1B).

**Immune responses induced by BCG vaccination in sensitized and naive mice.** We continued by investigating the immune response induced by BCG in sensitized and age-matched naive control CBA/J mice. ELISPOT was used to monitor frequencies of BCG-specific T cells before and 3, 5, 8, and 11 weeks after the s.c. vaccination with BCG (Fig. 2). Before BCG vaccination, no mycobacterium-specific IFN-γ-producing T cells were detected in any of the mice. Three weeks after BCG inoculation, the number of BCG-specific IFN-γ-producing cells in the draining lymph nodes had increased and reached the same level in sensitized and naive mice (Fig. 2A). The response in sensitized mice was, however, transient, and from 5 weeks after BCG inoculation and onwards, a higher frequency of mycobacterium-specific cells was found in naive vaccinated mice. At the termination of the experiment (week 11), a 10-times-higher frequency of BCG-specific T cells was found in the naive vaccinated group than in the sensitized vaccinated group (*P* = 0.032). A similar dynamic development of responses was found in the blood, although it was delayed so

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**FIG. 1.** BCG multiplication is inhibited in mice previously sensitized with environmental mycobacteria. (A) CBA/J mice. (B) C57BL/6J mice. The growth of BCG was compared in naive mice (open symbols) and in sensitized mice (solid symbols). The data shown are the means of BCG CFU ± standard errors. For both groups, five animals were sacrificed for each time point. The experiment was repeated twice with similar results.

**FIG. 2.** Influence of previous sensitization with environmental mycobacteria on the BCG-specific immune responses. BCG was administered s.c., and the frequencies of IFN-γ-producing cells isolated from the draining lymph nodes (A) and the blood (B) in naive mice (open symbols) and sensitized mice (solid symbols) were detected by the ELISPOT assay postvaccination after in vitro stimulation with BCG-Ag. The data presented here represent the logarithmic mean of results obtained from lymph node cells from three individual mice per group ± standard errors. The responses in the blood were analyzed on pooled cells from three animals for each time-point. A pilot experiment conducted on weeks 2, 4, and 6 supported the overall difference in the response profiles of the two groups of animals.
that higher frequencies of specific T cells were found from week 8 onwards in naive vaccinated mice (Fig. 2B). At no time point after vaccination was IL-4 or IL-5 detected in the supernatants of the stimulated cultures (results not shown).

**Sensitization with environmental mycobacteria blocks the protective effect of BCG, but not a TB subunit vaccine.** We continued by vaccinating sensitized and naive age-matched control CBA/J mice 4 to 5 weeks after the end of chemotherapy-treatment, followed 2 months later by an aerosol challenge with *M. tuberculosis*. The mice were killed 6 weeks post-TB infection, and *M. tuberculosis* CFU were enumerated in the lungs and spleens. The BCG vaccine imparted appreciable protection to naive mice against the TB challenge, with significantly reduced bacterial numbers in the organs (0.68 to 1.13 log₁₀ reduction; Table 1). Sensitization with environmental mycobacteria on its own, or followed by BCG vaccination, failed to induce a statistically significant level of protection against TB (Table 1).

We also asked if a previous sensitization with environmental mycobacteria would influence protection induced by a subunit vaccine. Groups of naive and sensitized CBA/J mice were vaccinated with BCG or injected (three times at 2-week intervals) with recently developed TB subunit vaccines based on the immunodominant antigens ESAT-6 and Ag85B mixed with a DDA-MPL adjuvant emulsion (5, 26). ESAT-6-vaccinated animals mounted a very strong recall immune response (5 to 7 ng of IFN-γ/ml) to the homologous preparation 1 week postvaccination in the blood (data not shown). The protection obtained by BCG in control mice was log 0.53, and as in the previous experiment, BCG did not protect presensitized mice (Table 2, experiment 1) The ESAT-6 subunit vaccine, in contrast, induced a similar degree of protection in both naive and sensitized mice. A subunit vaccine based on a fusion protein of Ag85B and ESAT-6 has recently been demonstrated to induce levels of protection similar to those of BCG in the mouse model (26), and this vaccine also protected against TB challenge at the same level in naive and sensitized mice (Table 2, experiment 2).

**Mycobacterial species isolated in Karonga, Malawi, differ in their ability to block BCG activity.** We investigated six different isolates from soil and sputum samples from Karonga District in Northern Malawi in the mouse model. Three of these isolates were typed as *M. fortuitum*, one was a strain of *M. chelonae*, and two were classified as belonging to the *M. avium* complex (Fig. 3). The growth of these isolates in spleen, liver, and lung was investigated with C57BL/6J mice over a period of 30 days. Most of the isolates were rapidly cleared to below the level of detection, but the strains from the *M. avium* complex multiplied and reached bacterial numbers 3 logs above those of *M. chelonae* and *M. fortuitum* after day 14 (Fig. 3). The mice...
were treated with chemotherapy, followed by an injection of BCG according to our standard protocol. BCG counts in the spleen of these mice were quantified at week 2 postinoculation (Fig. 4A). M. fortuitum and M. chelonae did not inhibit the growth of BCG, whereas bacteria from the M. avium complex reduced BCG numbers by 1 to 1.5 log (P < 0.01). This difference correlated with the immune responses induced by the BCG vaccine. There was no influence on the level of IFN-γ responses to BCG Ag by sensitization with M. chelonae or M. fortuitum, whereas the previous inoculation with bacteria from the M. avium complex completely ablated BCG immune responses (Fig. 4B). All strains, on the other hand, induced low and variable responses to antigens extracted from the homologous strain of environmental mycobacteria (results not shown).

**DISCUSSION**

This study demonstrates that animals exposed to certain environmental mycobacteria raise an immune response that controls the multiplication of BCG, thereby curtailing the vaccine-induced immune response before it is fully developed. The finding is important for the long-held discussion on the failure of BCG vaccination against TB in some parts of the world (15, 16, 38). One hypothesis to explain the failure of BCG was presented in 1966 by Palmer and Long, based on large-scale guinea pig experiments. They argued that contact with nontuberculous bacteria offers some level of protective immunity to TB, the protective effect of a superimposed BCG vaccine would be masked (33). The present study confirms the classical observation that priming with environmental mycobacteria promotes some levels of protective immunity to other mycobacteria (7, 10, 14, 33), in this case to BCG. However, this effect was not sufficient to significantly reduce the growth of M. tuberculosis, which multiplied at an almost unchanged rate in these sensitized animals. The difference from the partial protection imparted by environmental mycobacteria in the guinea pig model (14, 33) may be related to the fact that the earlier studies made no effort to clear the environmental mycobacteria by chemotherapy before challenge with M. tuberculosis, as well as the different genetic makeup and susceptibility of mice versus guinea pigs. The differences in these models and their relevance to human disease are the subject of an ongoing study.

That prior sensitization to environmental mycobacteria interferes in a similar way with human BCG vaccination is
strongly suggested by a number of classical epidemiological observations: (i) the finding of strong efficacy of BCG in trials in which tuberculin skin test-positive (and therefore sensitized) donors have been vigorously excluded (19); (ii) the consistent success with BCG in neonates vaccinated before any significant sensitization from environmental mycobacteria occurs (1, 9, 24); and, (iii) finally, the observation of a lower rate of skin test conversion, much smaller average diameter, and rapidly waning responses after BCG vaccination in areas with environmental sensitization (India and Egypt), compared with those in areas with minimal environmental exposure (Denmark) (4, 32). This observation was recently confirmed and extended by the observation of only minimal in vitro IFN-γ responses to purified protein derivative (PPD) induced by BCG vaccination in donors from Karonga, Malawi, compared to those from the United Kingdom (P. E. Fine and H. Dockrell, personal communication). Taken together, these findings are in agreement with the low and transient immune response in the group of animals sensitized with environmental mycobacteria before vaccination, whereas the naive animals developed strong and sustained responses (Fig. 3). Our experimental model is therefore relevant to the many tropical regions where BCG is not protective against pulmonary TB and where the high incidence of TB indicates that any partial protection provided by exposure to environmental mycobacteria is insufficient for the prevention of TB.

Our main conclusion is that BCG, as a live vaccine, is particularly sensitive to the influence of preexisting immune responses to antigens shared with certain environmental strains. In this regard, a recent study has demonstrated the cross-recognition of a large number of antigens shared between \textit{M. avium} and BCG (T. Pais and R. Appelberg, unpublished results). Multiplication is a precondition for the induction of immunity by BCG and killing of BCG by chemotherapy after administration has been demonstrated to abrogate subsequent immunity completely (13, 39). In the present study, this blocking is achieved by immunological control instead of chemotherapy, but the outcome in both cases is interference with the protective immune response, which would normally develop in response to the growing BCG. The requirement for BCG multiplication can be explained as a simple consequence of dosage, but more likely is due to the fact that only live BCG secretes many antigens of importance for the induction of a protective immune response (3, 28). Interestingly, our data from the animal model also suggest that only environmental strains, which are capable of an initial multiplication in the host, block the activity of BCG. A detailed evaluation of a large number of different soil isolates from Karonga, Malawi, and of their interactions with BCG is ongoing. In the future, information on the geographical distribution of such strains would be a valuable resource when trying to understand the huge variation in BCG efficacy in human trials.

This inhibitory effect of the environmental mycobacteria on the growth and activity of BCG provides an important argument in the ongoing discussion of live attenuated vaccines versus nonviable subunit vaccines against TB (12, 27, 44). In comparison with live attenuated vaccines, the present study suggests that subunit vaccines may be much less influenced by prior contact with environmental mycobacteria. As mentioned above, neonatal BCG vaccination consistently imparts protection against the childhood manifestations of TB (mostly extrapulmonary disease), but as its efficacy wanes over a period of 10 to 15 years (37), the adult pulmonary manifestations of TB are prevented neither by neonatal vaccination, by vaccination in adolescence after exposure to environmental mycobacteria (41), nor by a BCG revaccination strategy (22, 43). A TB subunit vaccine could therefore fulfill the criterion of having consistently high efficacy in different populations and may have a particularly important use for revaccination of third world children in adolescence.

ACKNOWLEDGMENTS

This study has been supported by the Danish Research Council and The European Commission (contract no. 18CT970254). Lise Brandt is supported by the Faculty of Health Science, University of Copenha-

REFERENCES

2. Andersen, A. B., Z.-L. Yuan, K. Hasløv, B. Vergmann, and J. Bennedsen. 1986. Interspecies reactivity of five monoclonal antibodies to \textit{Mycobacterium tuberculosis} as examined by immunoblotting and enzyme-linked immunosor- 


Editor: S. H. E. Kaufmann


