Failure of the *Mycobacterium bovis* BCG Vaccine: Some Species of Environmental Mycobacteria Block Multiplication of BCG and Induction of Protective Immunity to Tuberculosis

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The efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine against pulmonary tuberculosis (TB) varies enormously in different populations. The prevailing hypothesis attributes this variation to interactions between the vaccine and mycobacteria common in the environment, but the precise mechanism has so far not been clarified. Our study demonstrates that prior exposure to live environmental mycobacteria can result in a broad immune response that is recalled rapidly after BCG vaccination and controls the multiplication of the vaccine. In these sensitized mice, BCG elicits only a transient immune response with a low frequency of mycobacterium-specific cells and no protective immunity against TB. In contrast, the efficacy of TB subunit vaccines was unaffected by prior exposure to environmental mycobacteria. Six different isolates from soil and sputum samples from Karonga district in Northern Malawi (a region in which BCG vaccination has no effect against pulmonary TB) were investigated in the mouse model, and two strains of the *Mycobacterium avium* complex were found to block BCG activity completely.

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# 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-
INTERACTIONS OF BCG WITH ENVIRONMENTAL MYCOBACTERIA

**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>Log10 resistance</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>6.88*</td>
</tr>
<tr>
<td>Sensitization</td>
<td>4.36 ± 0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Sensitization + BCG</td>
<td>4.33 ± 0.17</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Naive or sensitized mice were BCG vaccinated (5 × 10⁸ CFU) followed by aerosol challenge with virulent *M. tuberculosis*.

**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 of three ATCC strains of environmental mycobacteria (*M. avium, M. scrofulaceum,* and *M. vaccae*). To clear the remaining mycobacteria, sensitization was followed, 3 weeks after the last inoculation, by 1 month of treatment with rifampin (Sigma; 100 mg/liter), ethambutol (Sigma; 200 mg/liter), and clarithromycin (Abbott Laboratories, Solna, Sweden; 200 mg/liter) added to the drinking water.

**TABLE 2. Bacterial numbers in organs of naive and sensitized mice after vaccination and aerosol challenge with virulent *M. tuberculosis***

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Spleen</td>
</tr>
<tr>
<td>Naive</td>
<td>6.36 ± 0.08</td>
<td>4.71 ± 0.05</td>
</tr>
<tr>
<td>BCG</td>
<td>5.89 ± 0.06</td>
<td>0.53*</td>
</tr>
<tr>
<td>DDA-MPL</td>
<td>5.34 ± 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>
</tbody>
</table>

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

**Protein concentration and enzymatic activity**

Protein concentration was quantified by the Micro bichromonic acid method (Fawcett, Rockford, Ill.).

Recombinant ESAT-6 was produced as described previously (18). The LPS content was below 0.3 ng/µg of protein and had no influence on cellular activity. The fusion protein Ag85B–ESAT-6 was produced as described recently (26). The proteins were kept at −80°C until use.

**Lymphocyte cultures**

Lymphocytes from spleens and blood were isolated and cultured as described previously (5). Briefly, cells from individual mice were cultured in microtiter wells (96 well; Nunc, Roskilde, Denmark) containing 2 × 10⁵ cells in a volume of 200 µl of RPMI 1640 supplemented with 5 × 10⁻⁴ M 2-mercaptoethanol, penicillin-streptomycin, 1 mM glutamine, and 5% (vol/vol) fetal calf serum. Based on previous dose-response observations, BCG Ag and ESAT-6 were each used at 5 µg/ml in the cultures. Phytomagglutinin at a concentration of 1 µg/ml was used in all experiments as a positive control for cell viability. IFN-γ, interleukin 4 (IL-4), and IL-5 were detected in 72-h culture supernatants by duplicate enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunospot (ELISPOT) analyses were conducted with cells from individual mice or, when blood was analyzed, with cells pooled from groups of mice, as described in reference 6. The detection level was 10 spots.

**Statistical methods**

Because all of the data show a normal distribution, the assessment of experiments was carried out by analysis of variance. Differences between means were assessed by Dunnett’s test (Tables 1 and 2) or Student's t test (see Fig. 2 and 4). A P value of <0.05 was considered significant.

***Sensitization with environmental mycobacteria***

Mice were immunized subcutaneously (s.c.) in the back three times at 2-week intervals with 2 × 10⁵ CFU of each of three ATCC strains of environmental mycobacteria (*M. avium, M. scrofulaceum,* and *M. vaccae*). To clear the remaining mycobacteria, sensitization was followed, 3 weeks after the last inoculation, by 1 month of treatment with rifampin (Sigma; 100 mg/liter), ethambutol (Sigma; 200 mg/liter), and clarithromycin (Abbott Laboratories, Solna, Sweden; 200 mg/liter) added to the drinking water.

**Vaccination**

A single dose of BCG Danish 1331 (5 × 10⁸ CFU) was injected s.c. at the base of the tail. There were no significant differences in the protection achieved with doses ranging from 5 × 10⁸ to 10⁹ BCG bacteria (results not shown). In one experiment, an l.v. dose of 5 × 10⁶ CFU of BCG was used to determine growth of BCG in naive versus sensitized mice. For subcutaneous vaccination, the mice were immunized s.c. three times at 2-week intervals with 10 µg (per dose) of either ESAT-6 or the Ag85B–ESAT-6 fusion protein emulsified in dioctadecylammonium bromide (DDA; 250 µg/dose; Eastman Kodak, Inc., Rochester, N.Y.) plus 25 µg of monophosphoryl lipid A (MPL; Corixa, Hamilton, Mont.) as described recently (5).

**M. tuberculosis infections**

Animals were infected with approximately 100 CFU of *M. tuberculosis* (Edman) per lung by the aerosol route in a Glas-Col inhalation exposure system. The mice were sacrificed 6 weeks after infection, and bacterial numbers in the lung and spleen were determined as described before (5).

The protective effect of BCG or subcutaneous vaccination was expressed as the log₁₀ reduction of the bacterial counts compared to that in the unvaccinated control mice. All results are based on five or six animals per group.

**Mycobacterial antigens**

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.
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 × 10^6 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).

**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 naïve 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.

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

**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 cells pooled 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

**FIG. 3.** Growth of isolates from Karonga, Malawi, in the mouse model. To evaluate the virulence of the isolates, mice were infected i.v. with *M. fortuitum* strains S160 (open circles), S78/2 (solid circles), and 2001 (solid triangles); *M. chelonae* strain 2015 (open triangles); and *M. avium* complex strains 1891 and 2011 (solid and open squares, respectively). The mycobacterial loads were determined in the liver, spleen, and lung at the time points indicated. *M. chelonae* and *M. fortuitum* were all below the level of detection from day 14 onwards. Data are given as means with standard errors (n = 4).
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 because 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 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.

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