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Efficacy of *Mycobacterium bovis* BCG Vaccination in Mice Undergoing Prior Pulmonary Infection with Atypical Mycobacteria

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The efficacy of *Mycobacterium bovis* BCG immunization in mice with established pulmonary infections caused by atypical mycobacteria was studied. In all four strains of *Mycobacterium* tested (*M. kansasii*, *M. simiae*, *M. avium*, and *M. scrofulaceum*), intravenous inoculation with 10^6 BCG had no discernible effect upon the course of atypical mycobacterial infection within the lungs; despite this, however, all BCG-vaccinated groups of mice were fully resistant to a subsequent acute aerogenic challenge with *M. tuberculosis* $H_{37}Rv$, regardless of the presence of the pulmonary atypical mycobacterial infections. Furthermore, animals infected with *M. kansasii*, *M. simiae*, or *M. avium* but not vaccinated with BCG expressed considerable antituberculous resistance within the lungs, resulting in significant prolonged survival of these animals. The relevance of these findings to the expression of antituberculous resistance in human populations in areas in which atypical mycobacteria are endemic and the failure of these findings to support the hypothesis that prior contact with atypical mycobacteria might in some way jeopardize or interfere with the efficacy of subsequent BCG vaccination are discussed.

It is becoming increasingly evident that atypical mycobacteria may play an important role in the pathogenesis of chronic tuberculous-lung infections (11). Despite this knowledge, however, little is known concerning the nature of the host immune response in vivo to atypical mycobacteria, particularly with regard to possible interactions between host cellular mechanisms generated by these organisms and the host cell-mediated immune response to *Mycobacterium bovis* BCG vaccination. It is clear that the possibility that prior infection with atypical mycobacteria might in some way interfere with and possibly jeopardize BCG vaccination processes is an area of considerable practical importance. Atypical mycobacteria are endemic to many areas of the world in which tuberculosis remains a serious problem, and the possible interaction between prior infection with these organisms and subsequent exposure to tuberculosis has long been recognized and explored (3, 7, 12). Moreover, the importance of such investigations has been further exemplified more recently by the failure of the extensive South India BCG vaccination trial, in which such possible interactions between the host response to atypical mycobacteria and the BCG vaccine has been put forward as a possible explanation for the apparent lack of efficacy of this vaccination program (4).

Youmans and his colleagues were among the first to examine the possibility that prior infection with various atypical mycobacteria might in some way cross-protect against subsequent *M. tuberculosis* infection (12). They showed that prior infection with certain strains of mycobacteria (*M. avium*, *M. kansasii*, and *M. intracellulare*) resulted in a significant reduction in mortality when mice were subsequently challenged with virulent *M. tuberculosis*. In contrast, prior infection with other mycobacteria (defined at that time as scotochromogens and rapid growers) gave no protection. An extensive study by Collins (1) subsequently confirmed these results and provided the additional information that antituberculous resistance in prior-infected mice seemed to depend upon the ability of the atypical mycobacteria to survive in vivo.

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It was clear, however, that, although these two reports provided convincing evidence to suggest that antituberculous resistance was raised in animals infected with atypical mycobacteria, both were based on the effect of infection with moderately high doses of atypical mycobacteria. Thus, neither study ruled out the possibility that a moderate atypical mycobacterial infection, established aerogenically within the lungs and thus at a site distant to that involved in the generation of acquired immunity to the BCG vaccine, might in some way interfere with this latter process (4, 8, 9). Moreover, in the earlier studies (1, 12), challenge with *M. tuberculosis* was also delivered intravenously, rather than by the more relevant aerogenic route. In this regard, therefore, the present study was designed to examine, within the limits imposed by an experimental model of natural infection, the possibility that the presence of established pulmonary atypical mycobacterial infections might in some way jeopardize the efficacy of subsequent BCG vaccination, as recently hypothesized elsewhere (8, 9), and hence reduce the subsequent resistance of the host to acute aerogenic challenge with *M. tuberculosis*. The results obtained reveal, however, that, although BCG vaccination had little effect on the course of the atypical mycobacterial infections within the lungs, in all cases tested the ability of the vaccinated animal to resist an aerogenic challenge with *M. tuberculosis* remained unimpaired.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female AB6 (A/Tru × C57BL/6) F_1 hybrid mice were used when 6 weeks of age. They were provided by the Trudeau Animal Breeding Facility, Saranac Lake, N.Y.

Bacteria. *M. bovis* BCG (Trudeau Mycobacterial Culture Collection strain 1011), *M. tuberculosis* $H_{37}Rv$ (102), *M. kansasii* (1203), *M. simiae* (1226), *M. avium* (724), and *M. scrofulaceum* (1306) were grown and stored as described elsewhere (1).

Experimental infections. Mice were aerogenically infected with atypical mycobacteria with a Middlebrook Airborne Infection Apparatus (Tri-R Instruments, Rockwell Center,

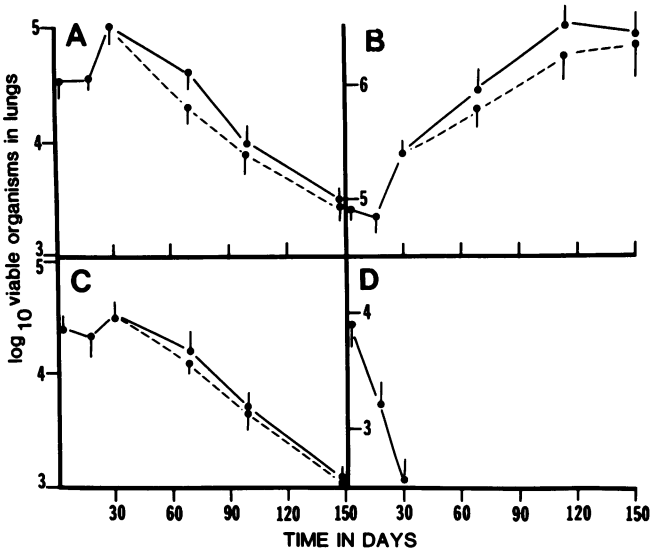

FIG. 1. Course of atypical mycobacterial infections with *M. kansasii* 1203 (A), *M. avium* 724 (B), *M. simiae* 1226 (C), and *M. scrofulaceum* 1306 (D) within the lungs of aerogenically infected mice. On day 30 of the initial infection, some animals (- - - - -) were inoculated intravenously with 1.2×10^6 viable *M. bovis* BCG Pasteur. Data are expressed as geometric means ± standard error of the mean ($n = 5$).

N.Y.) calibrated to deliver ca. 10^4 viable organisms within the lungs during the 30-min exposure period. After 30 days, groups of these mice plus appropriate controls were intravenously immunized with 10^6 viable BCG, followed after another 60 days by challenge of all animals plus controls with 10^4 *M. tuberculosis* delivered aerogenically as described above.

Enumeration of bacteria. The fate of the mycobacterial infections was followed by plating serial dilutions of individual homogenates of target organs on Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.) supplemented with 1 μg of 2-Thiophene-carboxylic acid hydrazide (Aldrich Chemical Co., Milwaukee, Wis.; the addition of this material selectively inhibits the growth of contaminating BCG), and bacterial colonies were counted after 14 to 20 days of incubation at 37°C. Organs from five randomly selected mice from each group were harvested at each time point; in the case of aerogenic infections, the uptake of bacteria within the lungs was determined 18 h after exposure and then at subsequent indicated time points.

Statistical method. Group comparisons were analyzed by the Student *t* test for unpaired data.

RESULTS

Lack of observable effect of intravenous BCG inoculation on the course of aerogenic atypical mycobacterial infections. The course of the aerogenic atypical mycobacterial infections over the 150-day period of a representative experiment is shown in Fig. 1. After a short period of growth over the first 30 days, both the *M. kansasii* and *M. simiae* infections slowly declined in numbers for the rest of the experimental period. In contrast, the *M. avium* infection grew progressively in the lungs for 120 days, after which there was some initial indication that this progressive growth had slowed or ceased. A further organism tested, *M. scrofulaceum*, rapidly declined in terms of numbers of viable organisms within the lungs, falling below levels of detectability after day 30.

In the case of the three persisting infections, *M. kansasii*, *M. simiae*, and *M. avium*, intravenous BCG immunization on day 30 of the experiment resulted in slightly fewer numbers of atypical mycobacteria being recovered from the lungs at subsequent time points; however, although this trend was observed in all three infected groups, the differences were statistically insignificant ($P < 0.10$).

Resistance to acute aerogenic tuberculous challenge. Two months after BCG immunization, all mice plus appropriate controls were exposed to acute aerogenic challenge with *M. tuberculosis* $H_{37}Rv$; the subsequent fate of this challenge infection is shown in Fig. 2. Normal mice exposed to the acute challenge died by day 30 from the progressive infection (mean survival time, 27 days), whereas in mice previously

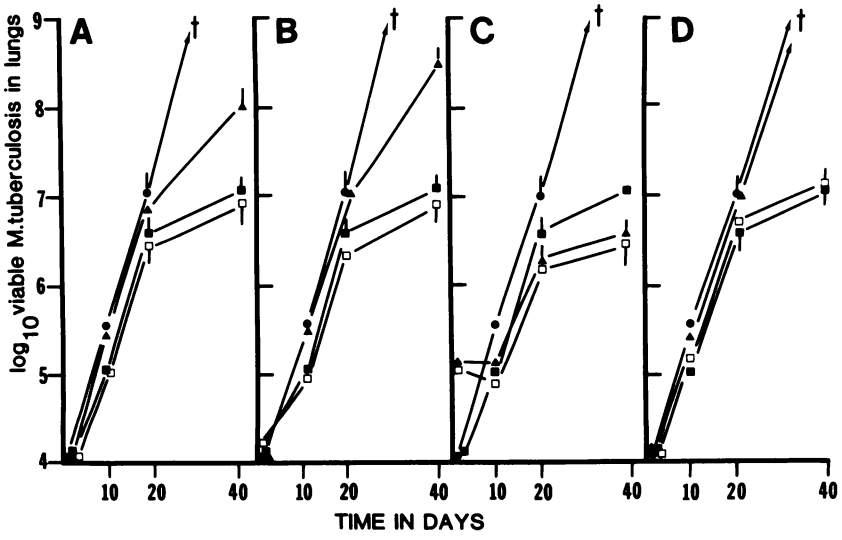

FIG. 2. Resistance of mice with pulmonary atypical mycobacterial infections with *M. kansasii* (A), *M. simiae* (B), *M. avium* (C), and *M. scrofulaceum* (D) to subsequent acute aerogenic challenge with virulent *M. tuberculosis*. The growth of *M. tuberculosis* in the lungs of normal controls (●), BCG-vaccinated controls (■), nonvaccinated prior-infected animals (▲), and vaccinated prior-infected animals (□) is expressed as the geometric mean ± standard error of the mean ($n = 5$).

TABLE 1. Prolonged survival after aerogenic tuberculous challenge in mice undergoing prior pulmonary atypical mycobacterial infections

Strain causing atypical infection[a]	Mean ± SEM survival time (days) of nonvaccinated controls (n = 6–7)
M. kansasii	78 ± 2.8
M. simiae	63 ± 2.0
M. avium	ND[b]
M. scrofulaceum	29 ± 2.5

[a] Exposure to atypical infections on day 1, followed 110 days later by exposure to *M. tuberculosis*.

[b] ND, not determined; five of seven animals still survived when the experiment was curtailed on day 100 of challenge.

immunized with BCG, the course of the *M. tuberculosis* infection was characterized by a period of progressive growth, followed by containment of the infection. Data obtained in this laboratory has shown that this containment process results in ca. 10^7 viable *M. tuberculosis* organisms being retained in the lungs, resulting in a persisting but apparently stable infectious state (Orme, manuscript in preparation).

The results shown in Fig. 2 provide two important pieces of information. First, they show that the presence of prior pulmonary infection with the tested atypical mycobacteria had no effect on the generation of acquired immunity to BCG, as evidenced by the demonstration that vaccinated prior-infected mice were fully resistant to the acute *M. tuberculosis* challenge. Second, the results confirm previous observations that the presence of atypical mycobacterial infections in nonvaccinated animals has the capacity in some way to confer upon the host increased resistance to tuberculous challenge (2, 7).

It was clear from these results, however, that increased resistance in the lungs of nonvaccinated mice varied between the atypical mycobacterial infections tested and that, furthermore, this resistance was apparently directly related to the course of the atypical mycobacterial infection itself. For example, in mice exposed to *M. simiae* or *M. kansasii*, in which the course of the infection in the lungs was characterized by a slow but progressive decline, a slowing of the progressively growing *M. tuberculous* challenge was observed in nonvaccinated mice (Fig. 2), resulting in prolonged survival of these animals (Table 1). In contrast, a different profile of events was observed in mice initially infected with *M. avium*; in these mice, the presence of the progressively growing *M. avium* infection created conditions within the lung which resulted in a substantially increased uptake of the *M. tuberculosis* challenge inoculum as compared with other groups of infected animals. Despite this increased uptake, however, substantial slowing of the growth of the *M. tuberculosis* infection was observed, regardless of whether the animals had been vaccinated with BCG. Indeed, both vaccinated and nonvaccinated *M. avium*-infected groups of mice showed increased resistance over that expressed by vaccinated controls ($P < 0.05$) on day 40 of the *M. tuberculosis* challenge infection.

Finally, in mice infected with *M. scrofulaceum*, in which the infection within the lungs rapidly declined, no evidence of increased resistance was observed within the lungs of nonvaccinated animals with these mice succumbing to the *M. tuberculosis* challenge within the same time span as normal controls.

Dissemination of atypical mycobacteria after aerogenic infection. After aerogenic infection with atypical mycobacteria, mice were monitored at various times to detect any dissemination of the infection to the spleen. In mice infected with *M. avium* (Fig. 3), dissemination of the infection to the spleen was observed on day 30, followed by progressive growth of the infection in the spleen for another 60 days. After this period, the course of the infection was characterized by an apparent bacteriostasis, with little or no change in the numbers of variable organisms, in similarity to that observed after intravenous infection (10). It was noted, however, that in mice receiving BCG on day 30, the containment of the *M. avium* infection in the spleen occurred somewhat faster, resulting in ca. 10-fold fewer organisms persisting in this organ by day 150 ($P < 0.05$).

A similar profile was observed in mice aerogenically infected with *M. kansasii*, although dissemination to the spleen was first detected much later during the experiment. Again, BCG vaccination apparently resulted in containment of this infection, although whether this was due to prevention of dissemination of the infection from the lungs or to accelerated containment of the infection within the spleen was unclear.

Finally, no dissemination of viable mycobacteria to the spleen was detected in mice aerogenically infected with either *M. simiae* or *M. scrofulaceum*.

Dissemination of *M. tuberculosis* after aerogenic challenge. After aerogenic challenge with *M. tuberculosis*, the dissemination of this organism to the spleen was also monitored. It was apparent (Fig. 4) that dissemination occurred in both vaccinated and nonvaccinated control groups, but with slowing and containment of the growth of the infection observable in mice immunized with BCG. Similar profiles were observed in mice initially aerogenically infected with *M. simiae*, *M. kansasii*, and *M. scrofulaceum*. In contrast,

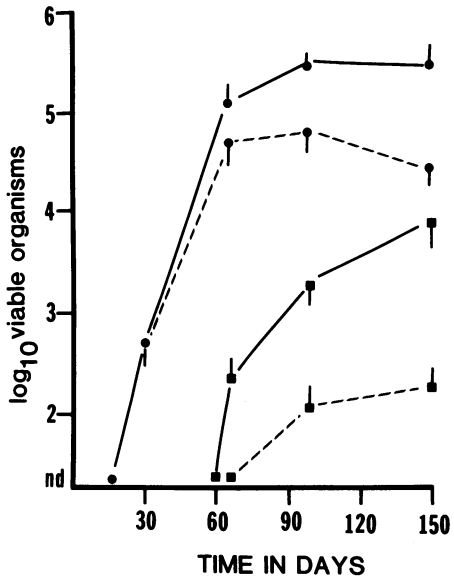

FIG. 3. Dissemination of *M. avium* (●) and *M. kansasii* (■) after aerogenic infection. The dissemination of the infection in infected controls (——) is compared with that in mice vaccinated with BCG on day 30 of the experiment (- - -). Data are expressed as geometric means ± standard error of the mean (n = 5). nd, Numbers of mycobacteria not determined (below level of sensitivity of assay [<1.8]).

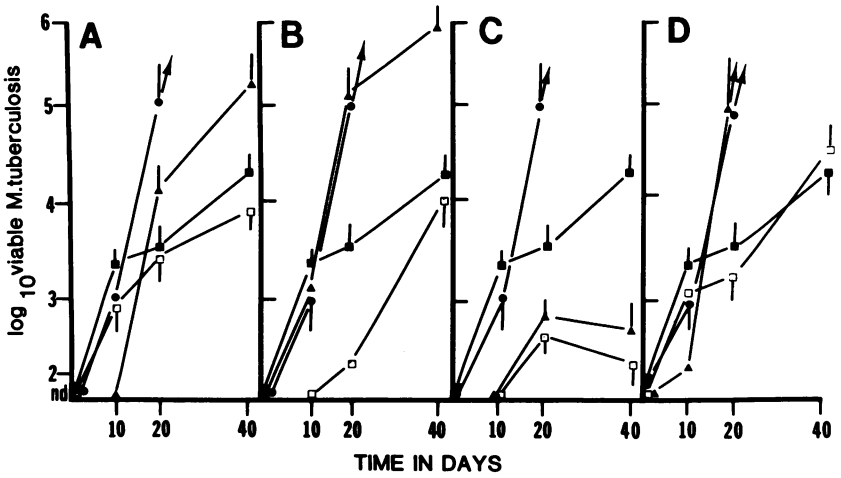

FIG. 4. Dissemination of *M. tuberculosis* to the spleen after acute aerogenic challenge with *M. kansasii* (A), *M. simiae* (B), *M. avium* (C), and *M. scrofulaceum* (D). The growth of *M. tuberculosis* in the spleens of normal controls (●), BCG-vaccinated controls (■), nonvaccinated prior-infected animals (▲), and vaccinated prior-infected animals (□) is expressed as the geometric means ± standard error of the mean (n = 5). nd, Numbers of mycobacteria not determined (below level of sensitivity of assay [<1.8]).

however, mice infected with *M. avium*, in which the course of infection was characterized by progressive growth within the lungs and early dissemination and growth within the spleen, only minimal dissemination of *M. tuberculosis* $H_{37}Rv$ was observed. Furthermore, no progressive growth on the *M. tuberculosis* infection was observed in the spleen, regardless of whether mice had been immunized with BCG.

DISCUSSION

This study has shown that the presence of established pulmonary atypical mycobacterial infections did not impair the capacity of an intravenous inoculum of BCG to immunize such animals against a subsequent acute aerogenic challenge with *M. tuberculosis*. Indeed, vaccinated prior-infected animals were able to control the growth of the *M. tuberculosis* challenge within the lungs in a manner similar to that observed in vaccinated control animals. Moreover, this study confirms previous observations (2, 7) that the presence of atypical mycobacterial infections can confer upon the nonvaccinated animal a state of increased resistance to tuberculous challenge and provides additional information which suggests that the magnitude of increased resistance expressed within the lungs may be directly related to the nature of the course of the atypical mycobacterial infection under test.

At first sight, the finding that BCG-vaccinated animals were able to control and contain a tuberculous challenge without this vaccination making an observable impact upon the course of the atypical mycobacterial infections is somewhat paradoxical. However, we have demonstrated elsewhere (6) that certain atypical mycobacteria, particularly those associated with persistent disease in the host, are intrinsically resistant to host bacteriostatic and bactericidal mechanisms, and hence, despite the propensity of these organisms to substantially activate host macrophages (Orme, in preparation), exhibit little change in growth patterns in the face of such host mechanisms. Further evidence in support of this hypothesis was provided by the present study, in which BCG immunization, a process that substantially activates host macrophages (5), had no significant detectable effect on the course of the atypical mycobacterial infections within the lungs.

The results in the present study are comparable with those in the report of Edwards et al. (2), who, in a guinea pig model, have provided convincing evidence that *M. avium* infection can confer significant protection against both $H_{37}Rv$ and a Southern India variant strain of *M. tuberculosis*. However, in their experiments, *M. avium* was delivered by the intradermal route, and hence it cannot be assumed that the observed antituberculous protection in the present study was mediated by an identical mechanism. It was clear, however, both in that report and in the present study, that the observed results did not support the hypothesis proposed by Rook et al. (8) that prior contact with atypical mycobacteria might in some way jeopardize or interfere with the generation of acquired cell-mediated immunity resulting from BCG immunization, and hence provide an explanation to account for the failure of the recent South India BCG vaccination trial (4).

It is recognized that laboratory experiments under controlled conditions with models involving acute aerogenic challenge obviously differ from conditions existing in the field. It is clear, however, that such experiments, designed to produce maximally observable effects, produce results which are comparable to those observed in low-dose infection models (2). A further aspect which must also be considered, however, concerns the possible effects of infection with atypical mycobacteria which do not produce persistent disease in experimental models; an obvious example is *M. scrofulaceum*, which can cause active disease in humans, particularly children (11), but apparently not in mice (1). The possibility remains, therefore, that this pathogen may have some effect on BCG vaccination, and this possibility should be addressed before the interference hypothesis of Rook et al. (8) can be completely ruled out.

In this regard, however, there are a number of hypotheses other than that of Rook et al. which have been put forward to explain the failure of the South India BCG vaccination trial. Among them, it has been suggested (4) that the high levels of environmental mycobacteria endemic to the area may act to raise the overall level of antituberculous resistance within the nonvaccinated population, so that a statistically significant increase in resistance within the vaccinated population could not be observed. Evidence in support of this hypothe-

sis is provided by the present study, which has demonstrated that the presence of atypical mycobacteria in the lungs resulted in both increased resistance and prolonged survival in the nonvaccinated animals after a virulent *M. tuberculosis* challenge; in the case of *M. avium* infection in particular, there were no discernible differences in these parameters between the vaccinated and control groups. It is concluded from these results, therefore, that the presence of pulmonary atypical mycobacterial disease may increase the antituberculous resistance of the infected host and, furthermore, did not in this model result in the generation of cellular mechanisms which demonstrably diminished the efficacy of BCG vaccination.

ACKNOWLEDGMENTS

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