Effect of *Mycobacterium bovis* BCG Vaccination upon *Mycobacterium lepraemurium* Infection

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Mice were infected with 10^8 *Mycobacterium lepraemurium* in the footpad (unsuppressed mice), and some of these animals were concurrently given 10^9 heat-killed *M. lepraemurium* intravenously (suppressed mice). These groups of mice were preimmunized with 10^7 viable organisms of *Mycobacterium bovis* BCG by several routes. BCG inhibited the proliferation of *M. lepraemurium* in the unsuppressed mice, but not in the suppressed mice. In effect, the intravenous administration of heat-killed *M. lepraemurium* suppressed the immunity to *M. lepraemurium* that BCG vaccination had engendered. BCG did not protect normal mice against intravenous infection with *M. lepraemurium*. It appears that the inhibitory effect of BCG vaccination upon *M. lepraemurium* infection is due to cross-reactive immunity rather than to nonspecific immunity or immunopotential. Thus, the route of BCG vaccination was immaterial, and vaccination 12 weeks before *M. lepraemurium* infection was as beneficial as vaccination 4 weeks before infection. Moreover, spleen cells from *M. lepraemurium*-immunized mice conferred adoptive immunity to BCG. The implications of this study for the use of BCG as a prophylactic and therapeutic agent in human leprosy are discussed.

In an earlier report, it was shown that concurrent or pretreatment of mice with heat-killed *Mycobacterium lepraemurium* intravenously (i.v.) enhanced footpad infection with *M. lepraemurium* due to suppression of cell-mediated immunity (11). Vaccination i.v. of such mice with 10^7 organisms of *Mycobacterium bovis* BCG appeared to block the suppressive effect of heat-killed *M. lepraemurium* i.v.

Three possible modes of action of BCG were considered. First, BCG might be acting as a nonspecific immunopotentiator of cell-mediated immunity, much as it does in the sheep erythrocyte system (14). Second, BCG infection might inhibit proliferation of *M. lepraemurium* by inducing enhanced macrophage microbicidal activity (2, 13). Finally, BCG might generate a cell-mediated immune response that cross-reacts with *M. lepraemurium*, since these mycobacterial species possess common antigens (4).

It has previously been shown that the role of BCG as a nonspecific immunopotentiator and inducer of macrophage activation is critically dependent upon its dose, route of administration, and interval between BCG and antigen injections (8, 14). By contrast, specific immunity is engendered by BCG after administration by various routes and doses, and immunity persists for long periods after immunization (10). Advan-

tage was taken of these differences to design experiments that would elucidate the mechanism by which BCG vaccination exerts its effect upon *M. lepraemurium* infection.

MATERIALS AND METHODS

Animals. (BALB/c $\times$ C57BL/6)F₁ inbred mice, which are highly susceptible to *M. lepraemurium* infection, were used (12).

***M. lepraemurium*.** The Hawaii strain was injected i.v. into passage mice. When the mice were moribund, they were killed, and *M. lepraemurium* bacilli were purified from the liver and spleen and stored at -70°C (12). Heat-killed bacilli were obtained by heating the living organisms to 100°C for 20 min, and bacterial clumps were dispersed with ultrasound.

There inocula of *M. lepraemurium* were used: 10^8 live bacilli were injected subcutaneously (s.c.) into the plantar surface of the left hind feet (LHF); 10^7 live bacilli were injected i.v.; and 10^9 heat-killed organisms were injected i.v. Mice that received live *M. lepraemurium* in the LHF and no heat-killed bacilli are referred to as "unsuppressed," whereas those that received 10^9 heat-killed *M. lepraemurium* i.v. are designated "suppressed."

BCG. The Pasteur strain (TMC 1011) was grown in Proskauer and Beck (PB) medium containing 2% glycerol and 0.1% Tween 80. Fresh-frozen culture of known viability was stored at -70°C . For immunization, BCG was injected either i.v., s.c. into the nape of the neck, or into the LHF, and the corresponding groups of mice are denoted by BCG (i.v.), BCG (s.c.), and BCG (LHF), respectively. The challenge dose of BCG was 10^5 i.v.

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***M. tuberculosis*.** The attenuated R1Rv strain (TMC 205) was grown in PB medium as described above, and stored at -70°C . Mice were challenged with 10^5 viable bacilli i.v.

Specific immunity. A total of 10^8 spleen cells from donors immunized with BCG or *M. lepraemurium* were injected i.v. into sublethally irradiated (500 rads) syngeneic recipients that were challenged with 10^5 BCG or R1Rv i.v. Two weeks later, the recipients were killed, and a viable count of BCG or R1Rv was made from each spleen homogenate by quantitative culture on Middlebrook 7H-10 agar containing 10 U of penicillin per ml (9). The geometric mean viable count was estimated for each group of 5 mice and expressed in $\log_{10}$ units. Immunity was estimated by subtracting the mean viable count of each cell recipient group from that of the control mice that received no spleen cells.

Nonspecific immunity. A mouse-virulent culture of *Listeria monocytogenes* was stored at -70°C . An inoculum of 10^5 viable bacilli was inoculated either i.v. or into the LHF. Twenty-four hours later the spleen or LHF was removed and homogenized, and the number of viable *L. monocytogenes* in the appropriate organ was determined by quantitative culture on phenylethyl alcohol agar. Immunity to *L. monocytogenes* was then estimated as described in the preceding paragraph for BCG and R1Rv.

Footpad swelling. The left and right hind foot thickness was measured with dial gauge calipers, and the left-right difference was expressed in millimeters.

Counts of *M. lepraemurium*. Organs were homogenized, and appropriate dilutions of homogenate were smeared on Reich slides (19), stained with phenol auramine rhodamine (22), and counted. The geometric mean count per group of mice was expressed in $\log_{10}$ units.

Statistics. Data were evaluated by analysis of variance, and group means were compared using the Q test (21).

RESULTS

Vaccination with BCG by different routes. Mice were vaccinated with 10^7 viable BCG either i.v., in the LHF, or s.c. at week -4. One month later (week 0), the distribution of viable BCG in the spleen and LHF was determined, nonspecific immunity to *L. monocytogenes* was measured in the spleen and LHF, and adoptive immunity to R1Rv was assayed in recipients of 5×10^7 spleen cells (Fig. 1).

As expected, there were a large number of viable BCG in the spleens of i.v.-immunized mice, and there were approximately 100-fold fewer BCG in the spleens of mice that were immunized by the s.c. and LHF routes. Nonspecific immunity to i.v. challenge was related to the number of viable BCG in the spleen; high resistance was found only in the animals immunized i.v. The mice that were immunized in the LHF had approximately 10^4 viable BCG in that site at week 0, and these animals were highly

FIG. 1. Immunological status of mice 4 weeks after i.v., s.c., or LHF (FP) vaccination with 10^7 viable BCG. Bar symbols: solid, viable BCG per spleen; horizontal broken, viable BCG per LHF; dotted, immunity to *Listeria* in the spleen; diagonal broken, immunity to *Listeria* in the LHF; cross-hatched, adoptive immunity to R1Rv in syngeneic recipients.

resistant to LHF challenge with *L. monocytogenes*. No BCG were recovered from the LHF of s.c.-immunized mice, but surprisingly, as many as 10^3 to 10^4 BCG were recovered from the LHF of i.v.-immunized mice. Neither of these groups of mice was resistant to LHF challenge with *L. monocytogenes*. Spleen cells from all groups of immunized mice conferred highly significant ($P < 0.01$) levels of specific immunity upon recipients, but the cells from the s.c.- and LHF-immunized mice were more effective ($P < 0.01$) than those obtained from i.v.-immunized mice. Evidently, the BCG-immunized mice were specifically sensitized at the systemic level, but nonspecific immunity was limited to those sites where the immunizing inoculum was originally implanted.

At week 0, groups of BCG-immunized and normal mice were infected with 10^8 *M. lepraemurium* into the LHF. Half of these mice were concurrently challenged i.v. with 10^9 heat-killed *M. lepraemurium*. At intervals thereafter for 24 weeks, the swelling of the LHF was measured, and the *M. lepraemurium* in the LHF and spleen were counted.

The effect of BCG immunization on the footpad swelling induced by *M. lepraemurium* is shown in Fig. 2. In mice not receiving heat-killed *M. lepraemurium* i.v. (Fig. 2A), BCG had no effect upon the early phase of footpad swelling at week 4. Subsequently, BCG-immunized mice, particularly those immunized by the s.c. or i.v. routes, had feet that were less swollen than unimmunized mice.

Heat-killed *M. lepraemurium* i.v. caused an

FIG. 2. Effect of i.v., s.c., and LHF vaccination with BCG upon LHF swelling induced by live *M. lepraemurium* in unsuppressed and suppressed mice. Symbols: (○) no BCG; (□) BCG i.v.; (△) BCG s.c.; (■) BCG LHF; (●) BCG LHF without *M. lepraemurium* infection. (A) Unsuppressed mice (10^8 live *M. lepraemurium* per LHF, no heat-killed *M. lepraemurium* i.v.); (B) suppressed mice (10^8 live *M. lepraemurium* per LHF, 10^9 heat-killed *M. lepraemurium* i.v.).

early suppression and late enhancement of the LHF swelling induced by live *M. lepraemurium* (Fig. 2B). Preimmunization with BCG completely neutralized the early suppressive effect of i.v. heat-killed *M. lepraemurium*. However, at later time points the LHF of BCG (s.c.)- and BCG (i.v.)-immunized mice were more swollen in mice that had received heat-killed *M. lepraemurium* than mice that had not. The pattern of LHF swelling was similar in BCG (LHF) mice, regardless of whether or not they had received heat-killed *M. lepraemurium*.

M. lepraemurium were counted in the LHF and spleens of mice 8, 16, and 24 weeks after infection (Fig. 3). It is apparent from Fig. 3A that BCG had an inhibitory effect on the growth of *M. lepraemurium* in the footpad. This effect was most marked in mice infected with BCG i.v., in whom it was evident at 8 weeks. Unlike our earlier experience (11), heat-killed *M. lepraemurium* i.v. did not increase the number of *M. lepraemurium* in the LHF of unimmunized mice (Fig. 3B), despite the gross increase in swelling. However, heat-killed *M. lepraemurium* i.v. substantially increased the number of *M. lepraemurium* per LHF in mice immunized with BCG ($P < 0.01$). This effect was most marked at 16 and 24 weeks. Once again, the growth of *M. lepraemurium* in the LHF was least in mice receiving BCG i.v. The *M. lepraemurium* con-

tent of the spleen was reduced in mice preimmunized with BCG (Fig. 4A), especially after LHF immunization. As with the LHF results, heat-killed *M. lepraemurium* did not enhance the spleen counts of unimmunized mice (Fig. 4B). Neither did it materially affect the counts in BCG (i.v.) and BCG (s.c.) mice; the apparent differences are not statistically significant. However, heat-killed *M. lepraemurium* caused a substantial increase of bacilli per spleen at 24 weeks in BCG (LHF) mice.

Influence of the interval between immunization and challenge. Mice were immunized with 10^7 BCG i.v. either 12 or 4 weeks before infection with 10^8 *M. lepraemurium* in the LHF and concomitant challenge with 10^9 heat-killed *M. lepraemurium* i.v. At the time of infection, the immunological status of the immunized animals was evaluated in terms of viable BCG per spleen, nonspecific immunity to i.v. challenge, and specific adoptive immunity (Fig. 5).

The mice immunized at week -12 had significantly lower numbers of BCG per spleen than mice immunized at week -4 ($P < 0.01$). The former mice had highly significant ($P < 0.01$) nonspecific immunity to *L. monocytogenes*, but less ($P < 0.01$) than that observed in the latter mice. Spleen cells from the two groups of BCG-immunized mice conferred a significant ($P < 0.01$) and closely similar level of adoptive immunity upon syngeneic recipients.

The LHF swelling resulting from *M. lepraemurium* infection is shown in Fig. 6. The early suppressive action of heat-killed *M. lepraemurium* i.v. was completely neutralized by BCG immunization, and subsequent to week 4, the LHF of BCG-immunized mice did not swell progressively in the absence of i.v. heat-killed *M.*

FIG. 3. Effect of i.v., s.c., and LHF vaccination with BCG upon multiplication of *M. lepraemurium* in the LHF of unsuppressed and suppressed mice. Symbols as in Fig. 2.

FIG. 4. Effect of i.v., s.c., and LHF vaccination with BCG upon growth of *M. lepraemurium* in the spleens of unsuppressed and suppressed mice. Symbols as in Fig. 2.

FIG. 5. Immunological status of mice 4 and 12 weeks after i.v. vaccination with 10⁷ BCG. Bar symbols: solid, viable BCG per spleen; dotted, immunity to *Listeria* in the spleen; horizontal broken, adoptive immunity to R1Rv in syngeneic recipients.

lepraemurium. However, in the presence of i.v. heat-killed *M. lepraemurium*, substantial LHF swelling ensued in BCG-immunized mice. The counts of bacilli in the LHF and spleen are shown in Fig. 7. Among mice that did not receive heat-killed *M. lepraemurium*, the counts of bacilli per LHF were lower in those mice that were vaccinated with BCG. Vaccination at -12 weeks appeared somewhat superior to that at -4 weeks, but the differences are not statistically significant. Higher counts of *M. lepraemurium* per LHF were obtained from mice given heat-killed *M. lepraemurium* i.v., a procedure that effectively abrogated the immunity conferred by BCG. The counts of bacilli per spleen are also shown in Fig. 7. It is apparent that at weeks 16 and 24, the number of bacilli per spleen was markedly lower ($P < 0.01$) in BCG-immunized mice, regardless of whether or not they received heat-killed *M. lepraemurium* i.v. Only in unimmunized mice did i.v. heat-killed *M. lepraemurium* increase the bacilli per spleen, but the increment was not statistically significant.

Induction of immunity to BCG after im-

munization with *M. lepraemurium*. The preceding experiments indicated that vaccination with BCG inhibited the growth of *M. lepraemurium* in the LHF and spleen, suggesting that cross-reactive immunity existed between these two mycobacterial species. Such cross-reactivity should be reciprocal, in which case mice infected with *M. lepraemurium* should also be immunized against BCG. This hypothesis was tested in the following experiment.

Groups of mice were infected with either 10⁸ *M. lepraemurium* or 10⁶ BCG into the LHF. At 2-week intervals, footpad measurements were taken of the LHF of *M. lepraemurium*-infected mice, and counts of bacilli per LHF were obtained every 4 weeks. Viable counts of BCG per LHF were obtained every 2 weeks from the other group of mice. Spleen cells were harvested from both groups of immunized mice, and 10⁸ lymphoid cells were injected i.v. into sublethally irradiated donors, which were challenged with 10⁵ BCG. Viable counts of BCG per spleen were

FIG. 6. Effect of i.v. BCG vaccination upon LHF swelling induced by live *M. lepraemurium* in unsuppressed and suppressed mice. Symbols: open, unsuppressed mice (10⁸ live *M. lepraemurium* per LHF, no heat-killed *M. lepraemurium* i.v.); closed, suppressed mice (10⁸ live bacilli per LHF, and 10⁸ heat-killed bacilli i.v.). Circles, No BCG; triangles, BCG i.v. at week -12; squares, BCG i.v. at week -4.

FIG. 7. Effect of i.v. BCG vaccination upon the multiplication of *M. lepraemurium* in unsuppressed and suppressed mice. Symbols as in Fig. 6.

made from cell recipients and controls 14 days later.

The *M. lepraemurium*-infected mice developed substantial footpad swelling by week 4, after which the rate of increase was slower (Fig. 8). There was a small but steady increase in *M. lepraemurium* bacilli per LHF. Viable BCG were steadily eliminated from the LHF during the 12-week period.

The adoptive immunity expressed by recipients of spleen cells is shown in Fig. 9. Cells obtained 2 weeks after infection with *M. lepraemurium* or BCG conferred a similar level of immunity ($P < 0.01$). Thereafter, cells from BCG-immunized donors were significantly ($P < 0.01$) superior. However, at each time point, spleen cells from *M. lepraemurium*-immunized donors conferred significant immunity ($P < 0.05$ or $P < 0.01$).

Effect of BCG vaccination against i.v. infection with *M. lepraemurium*. A group of 10 mice was immunized with 10^7 BCG i.v. at week -4. At week 0, these mice and 10 normal controls were given 10^7 *M. lepraemurium* i.v. The survival of these groups of mice was followed until all had died. From week 17 of i.v. *M. lepraemurium* infection the mice began to die, and the cumulative mortality is shown in Table 1. Evidently, immunization with BCG did not prolong the survival of mice challenged i.v. with *M. lepraemurium*.

DISCUSSION

In a previous study it was shown that a large dose of heat-killed *M. lepraemurium* i.v. enhances the local and systemic proliferation of live *M. lepraemurium* in mouse footpads, and that this effect is neutralized by vaccination with live BCG (11). It was thought that the beneficial effects of BCG might be due to cross-reactive immunity resulting from the considerable sharing of antigens between mycobacterial species (4), or that BCG might be acting as a nonspecific immunomodulating agent (14) or be generating nonspecific antibacterial immunity (13).

The specific and nonspecific effects of BCG can be easily differentiated. Specific immunity may be induced by a wide range of inocula administered by almost any route, and, beyond

FIG. 8. Vaccination of mice with 10^6 BCG and 10^8 *M. lepraemurium* in the LHF. Symbols: (○) smear counts of *M. lepraemurium* per LHF; (□) viable counts of BCG per LHF; (●) swelling of LHF in *M. lepraemurium*-infected mice.

FIG. 9. Adoptive immunity to R1Rv conferred by 10^6 spleen cells from BCG-vaccinated (solid bars) and *M. lepraemurium*-vaccinated (dotted bars) mice. All levels of immunity are highly significant ($P < 0.01$) except for the value indicated by the dot, which is significant at $P < 0.05$.

TABLE 1. Vaccination of mice with BCG against i.v. infection with *M. lepraemurium*

Weeks after infection	Cumulative mortality	
	10 ⁷ <i>M. lepraemurium</i> i.v.	10 ⁷ BCG i.v. plus 10 ⁷ <i>M. lepraemurium</i> i.v.
17	1	0
18	2	1
19	2	6
20	2	9
21	10	9
22		10
Median survival time (weeks)	20.5	19

a minimum time, the interval between immunization and challenge is immaterial (10). On the other hand, successful immunopotential and nonspecific immunity are exquisitely dependent on the dose of BCG, its route of administration, and the interval between vaccination and antigen injection (8, 14).

This study has revealed that BCG vaccination inhibits the proliferation of *M. lepraemurium* in the footpad and also inhibits its dissemination to the spleen. This result confirms a report of other investigators that BCG successfully immunized rats against s.c. (5) or corneal (17) infection with *M. lepraemurium*. Such protection of mice against *M. lepraemurium* infection by BCG is suggestive of specific cross-reactive immunity, an idea that is supported by the observations that BCG immunization by several routes was protective and that vaccinations given 4 and 12 weeks before challenge were of equal efficacy. Furthermore, there was no association between protection against *M. lepraemurium* and the level of immunity to *Listeria* in the spleen and footpad, a finding that argues against nonspecific resistance as the mechanism of protection. However, the spleen cells of all the mice vaccinated with BCG conferred adoptive immunity to *M. tuberculosis*, indicative of specific sensitization.

The notion that BCG vaccination induced a degree of specific immunity against *M. lepraemurium* infection was tested in the experiment in which spleen cells from *M. lepraemurium*-infected mice were transferred to recipients that were challenged with BCG. This experiment is based on the concept that cross-reactive immunity should be reciprocal in its expression. In practice, significant anti-BCG cross-reactive immunity was transferred by spleen cells from *M. lepraemurium*-infected mice, but the level of

adoptive immunity was markedly lower than that conferred by BCG-sensitized cells.

The suppression of specific immunity to *M. lepraemurium* has three features. First, the early phase of footpad swelling (at week 4) is inhibited. Later (after week 12), the footpad swelling is enhanced, and this, in turn, is associated with increased numbers of mycobacteria in the footpad and spleen (11). The first two features of suppression were consistently observed in this study, but the last was not. No satisfactory explanation can be offered for the discrepancy between enhanced footpad swelling and the absence of proportionately increased numbers of *M. lepraemurium* in those feet. A sample of smears was also stained by the Ziehl-Neelsen method (19), but the results were similar to those obtained with fluorescent dye. It is conceivable that the periodic acid-pararosaniline method (6, 15) that stains chromophobic mycobacteria may have been helpful. However, it should be borne in mind that the staining of mycobacteria is variable and beset with pitfalls (6, 15), and the staining procedures do not readily permit the distinction of dead from living organisms.

The outcome of the interaction of BCG and heat-killed *M. lepraemurium* upon the *M. lepraemurium* footpad infection was intermediate between the effects of BCG alone and heat-killed *M. lepraemurium* alone. The protective action of BCG and the suppressive action of heat-killed *M. lepraemurium* cancelled each other out. The net result was an animal that responded as if infected with *M. lepraemurium* alone, as judged by footpad swelling, bacilli per footpad, and bacilli per spleen. BCG given by the i.v. route neutralized the effect of heat-killed *M. lepraemurium* most effectively, suggesting that some local event in the spleen, where the BCG lesions and heat-killed bacilli would interact, may play a role in the prevention of suppression.

It was earlier found that BCG vaccination did not prolong the survival of mice challenged i.v. with 10⁹ *M. lepraemurium* (11). This disappointing result was attributed to the overwhelming magnitude of the challenge infection. However, in this study, BCG vaccination failed to protect against a more modest i.v. dose of 10⁷ *M. lepraemurium*. The presumptive reason for the failure of vaccination against i.v. challenge is that even modest doses of i.v. antigen suppress specific cell-mediated immunity, regardless of whether it is generated by the homologous or the cross-reacting organism.

The study is relevant to the situation of human leprosy because *M. leprae*, like *M. lepraemurium*, shares antigens with BCG (7). Vac-

nation with BCG protects mice against *M. leprae* (20) and has a more variable protective function in humans (1, 3). Moreover, reciprocal vaccination with dead *M. leprae* immunized mice against BCG, much as *M. lepraemurium* infection does (16). BCG has also been used as an immunotherapeutic agent in lepromatous leprosy (18). This study indicates that, to the extent that BCG shares with *M. lepraemurium* and *M. leprae* antigens to which cell-mediated protective immunity is generated, BCG vaccination may be an effective prophylactic vaccine. However, if the host is tolerant to *M. leprae* antigens that are common to BCG, it is probable that the prophylactic and therapeutic effectiveness of BCG would be either substantially impaired or ablated.

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