Effect of Treatment with BCG on the Course of Visceral Leishmaniasis in BALB/c Mice

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Received for publication 27 September 1976

Intravenous inoculation of BCG was found to be both prophylactic and therapeutic in BALB/c mice against challenge with amastigotes of Leishmania donovani. Spleens and livers of mice inoculated with BCG maintained total parasite burdens at significantly lower levels when compared to controls. BCG administered intravenously 14 days prior to and on the same day of protozoan challenge was more protective than vaccine given 30 and 14 days prior to challenge. A level of 10⁷ viable units of BCG provided more protection against challenge with parasites than did 10⁶ viable units. BCG given the same route as the challenge dose of amastigotes provided more protection than if administered via some other route. BCG given to mice with an already established infection was shown to significantly reduce their parasite burdens.

Leishmaniasis, a disease caused by protozoa of the genus Leishmania Ross, 1903, manifests itself in three major forms: cutaneous, mucocutaneous, and visceral. Each form of leishmaniasis is caused by a different species of Leishmania.

Immunity in leishmaniasis appears to be cellular in nature (9), although cell-bound antibodies may influence the expression of this cellular response (7, 11, 20). In man, the induction of cell-mediated immunity depends upon the immunological competence of the host and the virulence of the infectious agent. The more virulent forms of organisms, those causing the most severe and rapidly healing disease, provide the best and most persistent immunity. For example, Leishmania tropica major causes a more severe disease than Leishmania tropica minor. An individual who has recovered from infection with L. tropica major is immune to re-infection with the latter organism; however, the reverse is not true. Individuals who have recovered from infection with L. tropica minor are not immune to infection with L. tropica major. This phenomenon is the basis for the massive vaccination program used in Russia (20, 27, 35).

To date no attenuated strains of Leishmania providing adequate protection have been found. In addition, there is no cross immunity between the various forms of leishmaniasis (18, 20, 29, 36, 39). As a result, leishmaniasis remains a menace in certain countries and continues to be a major world health problem.

Complement-fixing antibodies produced by kala-azar (visceral leishmaniasis) patients are best demonstrated with the use of Mycobacterium antigens (16, 25, 26, 34, 38, 42). Even though these antibodies have no detectable role in immunity against the disease, the use of Mycobacterium antigen in the complement-fixation test for kala-azar indicates a specific cross-reactivity between members of the protozoan genus Leishmania and the bacterial genus Mycobacterium.

In this paper, we report the effects of inoculation of BALB/c mice with BCG upon the growth of Leishmania donovani in the spleens and livers of the recipients.

MATERIALS AND METHODS

Animals. Female BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, Me., and Charles River Breeding Laboratories, North Wilmington, Mass. These were used when they were 6 weeks old and weighed 16 to 20 g. Golden hamsters (Mesocricetus auratus) were originally obtained from the Rocky Mountain Laboratory, Hamilton, Mont. They were maintained and bred in the laboratory facilities of the Department of Microbiology, University of Montana.

L. donovani. Hamsters infected with the Sudan III strain of L. donovani were obtained from Leslie Stauber, Rutgers University, New Brunswick, N.J.

Vaccine. Mycobacterium bovis, strain BCG, was maintained in the Stella Duncan laboratory from a culture that had been obtained from the Pasteur Institute, Paris, France. The stock vaccine was held at -70°C.

Giemsa stain. Giemsa stain was used for staining all impression smears of spleens and livers.

Determination of total parasite burden. The de-
termination of the total parasite burden of *L. donovani*-infected tissue was done by the methods suggested by Stauber (40, 41). A small piece of infected spleen or liver tissue was blotted on a paper towel to remove blood and loose cells. The cut face of the tissue was dabbed lightly on an alcohol-cleaned slide. At least 15 to 25 impression smears were made for each organ assayed. The slide was air-dried, fixed for 10 s with absolute methanol, and stained for 30 to 60 min with Giemsa stain. The impression smears were studied with light microscopy. The ratio of amastigotes/cell nuclei was determined as follows. While the impression smears were being scanned with an oil immersion magnification (×1,000), a count of the amastigotes and cell nuclei was made. As soon as one of the two counts reached 1,000, the ratio of amastigotes/cell nuclei (LD/CN) was calculated. This ratio, when multiplied by the weight of the organ or tissue (in milligrams) and multiplied by 200,000, gives a value that is considered to be the estimated total parasite burden (TPB), e.g.,

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TPB = \frac{\text{number of amastigotes}}{\text{number of cell nuclei}} \times \text{weight of organ} \times 200,000
\]

**Maintenance of *L. donovani* in hamsters.** Serial passage of *L. donovani* was accomplished by intracardial inoculation of infected hamster spleen tissue containing amastigotes. An inoculum of 2.0 × 10⁸ parasites suspended in 0.2 ml of sterile Sorenson phosphate-buffered saline having a pH of 7.2 was used. The recipient was necropsied 30 to 40 days postinfection (p.i.), and the spleen was aseptically removed and weighed. The total parasite burden was determined, and the spleen was suspended in an appropriate dilution of sterile phosphate-buffered saline (pH 7.2) and ground in a Ten Broeck tissue grinder. The tissue suspension was filtered through sterile gauze pads to remove large aggregates of spleen tissue, and the filtrate, which contained the amastigotes, was used for transfer of the organisms to hamsters and mice.

**Statistical analysis.** All data presented in the figures are mean values. The vertical lines extending from the mean values represent two standard errors from the mean. *P* values were determined by Student's unpaired *t* test.

**RESULTS**

Determination of the effect of prior treatment with BCG on the course of visceral leishmaniasis in BALB/c mice. Separate groups of mice were inoculated intravenously (i.v.) with 1.0 × 10⁶ viable units (VU) of BCG either 30 and 14 days before or 14 days before and at the time of i.v. challenge with 2.0 × 10⁶ amastigotes of *L. donovani*. The mice receiving injections of only amastigotes were also treated with 0.2 ml of sterile Dubos broth 30 and 14 days before challenge with *L. donovani*. At 18 h and at 16 and 35 days p.i., 5 to 10 mice from each group were necropsied.

Mice inoculated i.v. with 2.0 × 10⁶ amasti-
inoculated i.v. with $2.0 \times 10^6$ amastigotes on day 0. Animals in groups C and D were challenged i.v. on day 0 with $2.0 \times 10^7$ amastigotes. Mice in groups B and D were inoculated i.v. with $1.0 \times 10^7$ VU of BCG 14 days before and on the day of challenge with *L. donovani*. Mice in groups A and C were injected i.v. with 0.2 ml of sterile Dubos broth on 14 days prior to and on the same day of protozoan challenge. At 30, 45, and 60 days p.i. seven mice from each group were necropsied.

Mice inoculated i.v. with $2.0 \times 10^6$ amastigotes had mean spleen parasite burdens of approximately $8.5 \times 10^5$ at 30 days p.i. (Fig. 3). This parasite burden was maintained over the next 30 days. Animals given 1-log-fewer parasites ($2.0 \times 10^5$) exhibited mean spleen parasite burdens of approximately $9.0 \times 10^4$ at 30 days p.i. This parasite burden gradually increased to $4.0 \times 10^6$ by 60 days p.i. Administration of $1.0 \times 10^7$ VU of BCG i.v. 14 days prior to and on the day of protozoan challenge reduced the parasite burdens of the spleens of both groups to prepatent levels. Both groups of infected mice inoculated with BCG had no detectable parasites in their spleens during the 60-day study period.

Figure 4 shows the parasite burdens of the livers of both control mice and mice that had been inoculated with BCG. Mice inoculated i.v. with $2.0 \times 10^6$ amastigotes had liver parasite burdens of approximately $2.4 \times 10^7$ 30 days p.i. The parasite burdens steadily declined to $4.3 \times 10^6$ by 60 days p.i. Administering BCG i.v. 14 days prior to and on the day of challenge ($2.0 \times 10^6$ amastigotes) significantly reduced the parasite burdens ($P < 0.01$). Three of the seven mice that had been given BCG and challenged with $2.0 \times 10^6$ amastigotes had reduced parasite burdens of $3.7 \times 10^6$. The remaining four mice had parasite burdens that were too low to detect. All mice that had been treated with BCG and challenged with $2.0 \times 10^6$ amastigotes had no detectable liver parasites 45 and 60 days p.i. Animals inoculated i.v. with 1-log-fewer amastigotes ($2.0 \times 10^5$) had liver parasite burdens of $1.3 \times 10^7$ at 30 days p.i. The parasite burdens declined to a mean value to $3.5 \times 10^6$ by day 45 p.i. and remained at that level for the duration of the experiment. Mice given BCG i.v. and challenged i.v. with $2.0 \times 10^5$ amastigotes had reduced parasite burdens at the prepatent level throughout the entire 60-day study period.

Determination of the immunotherapeutic effects of BCG on BALB/c mice inoculated with *L. donovani*. Eighty-four mice were di-
Animals similarly infected but treated with i.v. 15 days p.i. to mice had no detectable parasites and the other three mice had significantly reduced (P < 0.05) spleen parasite burdens.

Figure 6 shows the effect of BCG immunotherapy on the liver parasite burdens of BALB/c mice challenged i.v. with two different doses of *L. donovani*. Administering BCG i.v. on 15 and 31 days p.i. did not significantly reduce the liver parasite burdens of mice in either challenge group.

Determination of the effect of the route of BCG treatment on the course of visceral leishmaniasis in BALB/c mice. Sixty-three mice were divided into three groups of 21 mice each. Mice in group B were given 1.0 x 10^7 VU of BCG by the i.v. route, 14 days prior to and on the same day of protozoan challenge. Animals in group C were inoculated i.p. with a similar dose of BCG. Mice in group A were inoculated i.v. with 0.2 ml of sterile Dubos broth 14 days prior to and on the same day of protozoan challenge. All groups of mice were challenged i.v. with 2.0 x 10^6 amastigotes. Seven mice from each group were necropsied at 30, 45, and 60 days p.i.

Mice challenged i.v. with 2.0 x 10^6 amastigotes attained peak spleen parasite burdens in approximately 30 days (Fig. 5). Their spleen parasite burdens were maintained at this level over the next 30 days (60 days p.i.). One dose of BCG administered by i.v. 15 days p.i. to mice that had been challenged with 2.0 x 10^6 amastigotes did not significantly reduce their spleen parasite burdens by day 30, but a second dose of BCG given i.v. at 31 days p.i. resulted in a significant (P < 0.01) reduction in splenic parasites by day 45 and day 60. Mice inoculated i.v. with fewer (2.0 x 10^5) amastigotes had lower spleen parasite burdens during the 60-day study period than those mice that had been challenged with a 1-log-higher dose of parasites. Animals similarly infected but treated i.v. with BCG at 15 and 31 days p.i. had fewer
Each group were necropsied at 30, 45, and 60 days p.i.

Figure 7 shows the spleen parasite burdens of mice inoculated either i.v. or i.p. with BCG and challenged i.v. with $2.0 \times 10^6$ amastigotes of \textit{L. donovani}. Untreated mice attained spleen parasite burdens of $8.5 \times 10^6$ at 30 days p.i. This level of parasitism remained relatively constant during the next 30 days. Mice given BCG i.p. had significantly lower populations of amastigotes in their spleens at 30 and 45 days p.i. but not at 60 days p.i. when compared to controls. Animals inoculated i.v. with BCG and challenged i.v. with amastigotes had prepatent levels of splenic parasites throughout the 60-day study period.

Figure 8 shows the liver parasite burdens of mice inoculated i.v. or i.p. with BCG and challenged i.v. with $2.0 \times 10^6$ amastigotes. Infected mice reached a peak liver parasite burden of $2.4 \times 10^7$ amastigotes at 30 days p.i. The liver parasite burdens declined steadily to $4.3 \times 10^5$ by 60 days p.i. Mice inoculated i.p. with BCG and challenged i.v. with $2.0 \times 10^6$ amastigotes had liver parasite burdens equivalent to the control groups throughout the 60-day experiment. Animals given BCG i.v. and challenged i.v. with amastigotes had significantly fewer ($P < 0.001$) liver parasites than did the control mice. At 30 days p.i., three of the seven infected mice that were given BCG by the i.v. route had detectable liver parasites (mean, $3.7 \times 10^6$), and four of the seven mice had prepatent levels of liver parasites. Mice inoculated i.v. with BCG and challenged i.v. with amastigotes had no detectable parasites at 45 or 60 days p.i.

**DISCUSSION**

BCG, an attenuated strain of \textit{M. bovis}, has long been used as a nonspecific stimulator of the reticuloendothelial system (4-6). It has been noted that mice which were previously infected with \textit{Mycobacterium phlei}, BCG, \textit{Mycobacterium butyricum}, or two atypical isolates of mycobacteria from man supported significantly lower populations of \textit{L. donovani} than did the unvaccinated controls (F. C. Goble, E. A. Konopka, J. L. Boyd, and L. Lewis, Proc. 7th Int. Congr. Trop. Med. Malar. 2:238, 1963). Conversely, animals infected with \textit{L. donovani} resisted challenge with \textit{Mycobacterium tuberculosis}.

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**Fig. 6.** Mean total parasite burden of livers of BALB/c mice inoculated i.v. with varying doses of amastigotes of \textit{L. donovani} and treated i.v. with $1.0 \times 10^7$ viable \textit{M. bovis} (BCG) as indicated. Symbols:

- $2.0 \times 10^6$ amastigotes on day 0;
- $2.0 \times 10^8$ amastigotes on day 0 plus BCG 15 and 31 days p.i.;
- $2.0 \times 10^6$ amastigotes on day 0 plus BCG 15 and 31 days p.i.;

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**Fig. 7.** Mean total parasite burden in spleens of BALB/c mice inoculated i.v. with $2.0 \times 10^6$ amastigotes of \textit{L. donovani} and treated by various routes with $1.0 \times 10^7$ viable \textit{M. bovis} (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols:

- Amastigotes on day 0;
- Amastigotes on day 0 plus BCG i.v.;
- Amastigotes on day 0 plus BCG i.p.;

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and macrophages. It has been shown that administration of BCG to sensitized mice activates macrophages (17) and that injection of BCG protoplasm into mice sensitized with an oil-treated BCG cell wall vaccine results in the release of large amounts of soluble mediators into the blood stream (3). These factors would operate to prevent the establishment of a population of L. donovani in the spleens of mice given BCG 14 days prior to and on the same day of protozoan challenge. On the other hand, mice inoculated with BCG 30 and 14 days prior to infection also appeared to effectively control proliferation of protozoa in their spleens. Inoculation of BCG into mice produces tubercles, which, because of their histological structure, serve to produce a low but continuous release of soluble mediators. Growth of BCG in the spleens apparently ceases or is markedly reduced by 14 days after inoculation (R. V. Blanden, M. R. Lefford, and G. B. Mackaness, J. Exp. Med. 129:1079, 1969), resulting in a decreased release of lymphokines. As a result, a challenge dose of amastigotes given 14 days after administration of BCG may not be cleared from the spleen as effectively as it is in mice that were given the second dose of BCG on the day of challenge. The role, production, and effect of soluble mediators produced by sensitized lymphocytes during a cell-mediated immune response has been well established in the literature (8, 15). The presence and role of soluble mediators produced in response to leishmaniasis has also been reported (7, 9, 10, 43).

The inoculation of mice with 1.0 × 10⁶ rather than with 1.0 × 10⁷ VU of BCG results in greater protection against subsequent infection with L. donovani. Mackaness and Blanden (33) reported that increasing the number of BCG bacilli administered to guinea pigs and mice intensified their delayed hypersensitivity responses. The enhanced response of animals that were given greater numbers of BCG can be related to the greater number of organisms which survive and proliferate in the host and serve as antigen depots for stimulation of lymphocytes and for production of soluble mediators.

In our study of the immunotherapeutic effects of the administration of BCG to mice which had been previously infected with L. donovani, there was no effect upon the liver parasite burdens of mice treated by i.v. injections of BCG 15 and 31 days after infection with either 2.0 × 10⁶ or 2.0 × 10⁵ amastigotes. The effect of a secondary stimulation with BCG as well as the ability of BCG mice to control L. donovani infections of the spleen induced with different numbers of amastigotes is well illus-

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**Fig. 8.** Mean total parasite burden in livers of BALB/c mice inoculated i.v. with 2.0 × 10⁶ amastigotes of L. donovani and treated by various routes with 1.0 × 10⁸ viable M. bovis (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols: ■, Amastigotes on day 0; □, amastigotes on day 0 plus BCG i.v.; △, amastigotes on day 0 plus BCG i.p.; ———, patency level.
treated. A single dose of BCG failed to decrease the spleen parasite burden in mice that had been infected with 2.0 × 10^6 amastigotes, but a second dose resulted in a significant decrease in the number of organisms present in the spleen. The administration of BCG to mice infected with the lower number of protozoa effectively controlled the spleen parasite burdens among them. The failure of mice infected with larger numbers of amastigotes to respond as well to BCG therapy compared to those which had been injected with the smaller number of protozoa may result from immunological tolerance, antigenic competition, or paralysis of the reticuloendothelial system. This supposition is supported by the results of other workers. Adler (1) reported that hamsters infected with *L. donovani* for an extended length of time were unable to reject skin homografts, indicating an alteration of the cell-mediated mechanism in the animal. Bryceson et al. (9, 11) reported that i.v. injection of tolerogenic doses of purified soluble antigen prepared from *Leishmania enriettii* or injection of 1.0 × 10^6 or greater numbers of *L. enriettii* into the skin of normal guinea pigs results not only in an impairment of their development of delayed-type responses to leishmanin antigen, but also in a more severe disease which frequently resulted in death. These investigators also reported that multiple intradermal injections of guinea pigs with bacterial adjuvants such as Freund incomplete adjuvant with silica, killed *M. tuberculosis*, or killed *Corynebacterium parvum* prior to intracutaneous challenge with *L. enriettii* resulted in a more severe cutaneous infection, leading to metastasis and death. The pretreatment of these guinea pigs with the various adjuvants did not interfere with their development of cutaneous delayed hypersensitivity to leishmanin antigen but did induce the formation of anti-leishmanial antibodies. This observation was surprising, since antibodies were not normally produced by guinea pigs infected with *L. enriettii*. Clinton et al. (14) studied the antibody response to ovalbumin in hamsters infected with *L. donovani*. It is interesting to speculate on the possibility that the antibodies produced in adjuvant-treated animals may have acted as "blocking antibodies," interfering with the destruction of the parasites by activated macrophages. Allwood and Asherson (2) reported that the bacterial adjuvants mentioned above interfere with the migration of T-lymphocytes into lymph nodes, thus interfering with the induction phase of the delayed response. This may explain, in part, the increase in severity of infection in adjuvant-treated and infected guinea pigs, but it does not explain why the guinea pigs were able to exhibit a delayed-type response to the leishmanin antigen.

The route of injection of BCG is of major importance for the protection of mice inoculated with *L. donovani*. BALB/c mice that had been treated by i.v. injection of BCG and challenged i.v. with amastigotes had fewer spleen and liver parasites than did those mice that had been inoculated i.p. with BCG and challenged i.v. (Fig. 7 and 8). Larson et al. (30, 31) reported that administration of BCG to mice will protect against challenge with Friend Disease virus if the BCG is given via the same route as the challenge dose. The reasons for this are not clear, but one obvious explanation is that the distribution of acid-fast bacilli in the host differs as the route of administration of BCG is varied. The small granulomata (19) important to the activity of nonspecific resistance will, therefore, vary in their distribution. It is important that the reticuloendothelial cells stimulated with BCG be in the same area as those affected by the parasite.

There are at least three mechanisms by which BCG immunization produces its effect. The granulomata produced by administration of BCG are so structured as to be an ideal site for the production of soluble mediators. The central area of the granulomata contains actively metabolizing bacilli, and these release products that serve to stimulate lymphocytes in the peripheral area. The stimulation results in the synthesis of soluble mediators by the sensitized lymphocytes to perform a multitude of activities. Cytotoxic lymphocytes capable of destroying cells containing *L. donovani* could also be produced at granulomata sites. The findings of Evans and Alexander (17), Krahenbuhl and Remington (28), and Hibbs et al. (22, 24) suggest that macrophages are the cells that are ultimately responsible for the manifestation of nonspecific resistance. The results of these workers suggest that after inoculation with moderate doses of BCG, macrophages are "armed" and that almost immediately after administration of a second dose of BCG these cells become "activated." Such activated macrophages manifest the ability to destroy tumor cells and other cells with modified surface membranes. The destruction of these cells is brought about by membrane-to-membrane contact, and phagocytosis occurs only late in the course of killing the cells. The studies of Lodmell et al. (32) show that macrophages can destroy cells whose surface membranes are modified by the presence of herpesvirus. If soluble mediators released by sensitized T-cells activate macrophages, macrophages paradized with *L. donovani* or the parasites within
the activated macrophages should be destroyed more effectively if the mediators are released in the immediate area of infection in the spleen. The reduced clearance of protozoans from the liver cells of infected mice treated with BCG could thus be explained, since the liver is not an immunocompetent organ (37). The spleen, however, has an abundance of T-cells and macrophages possessing the capacity to affect cell-mediated immunity.

ACKNOWLEDGMENTS

We thank J. Smith and K. Olson for their technical assistance. We also wish to acknowledge final preparation of the manuscript by J. Green, K. Kelly, and J. Sanderson. This study was conducted in the facilities of the Stella Duncan Memorial Laboratories and was partially supported by Public Health Service training grant 5-T01-AI 00358 and research career award 4-AI-16502-11, both from the National Institute of Allergy and Infectious Diseases.

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