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

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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 10^7 viable units of BCG provided more protection against challenge with parasites than did 10^6 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 reinfection 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.,

$$\text{TPB} = \left(\text{number of amastigotes/number of cell nuclei}\right) \times \text{weight of organ} \times 200{,}000$$

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 2.0×10^6 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 1.0×10^6 viable units (VU) of BCG either 30 and 14 days before or 14 days before and at the time of i.v. challenge with 2.0×10^6 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 2.0×10^6 amastigotes of *L. donovani* attained a peak parasite burden in their spleens (mean, 1.0×10^7) in about 35 days (Fig. 1). Mice inoculated i.v. with BCG on 30 and 14 days prior to challenge with *L. donovani* had a spleen parasite burden similar to controls at 16 days p.i. However, at 35 days p.i., the spleen parasite burden of these mice was reduced to prepatent levels. Mice inoculated i.v. with similar doses of BCG 14 days prior to and on the day of challenge had no detectable parasites in their spleens at 16 or 35 days p.i.

The livers of mice inoculated i.v. with 2.0×10^6 amastigotes developed peak parasite burdens in approximately 16 to 35 days (Fig. 2), whereas those inoculated i.v. with BCG 30 and 14 days prior to challenge had fewer liver parasites at 16 days p.i. ($P < 0.001$) in comparison to control mice. In mice inoculated i.v. with BCG 14 days prior to and on the day of challenge, the liver parasite burden was also significantly reduced at 16 days p.i. ($P < 0.01$) and 35 days p.i. ($P < 0.005$).

Determination of the effects of prior treatment with a higher dose of BCG on the course of visceral leishmaniasis in BALB/c mice challenged with various doses of *L. donovani*. Eighty-four mice were divided into four groups of 21 mice each. Mice in groups A and B were

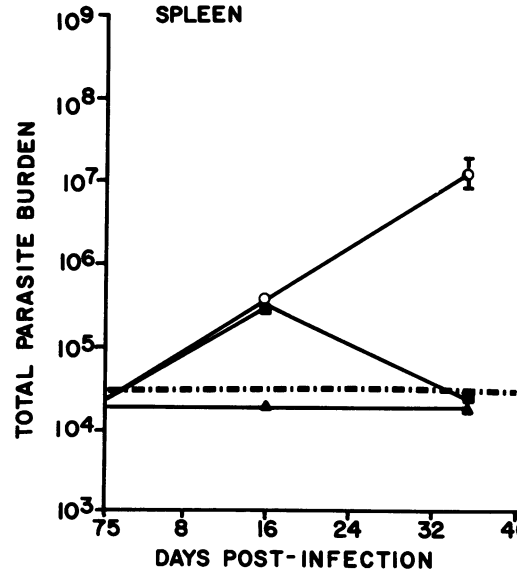

FIG. 1. *Mean total parasite burden in spleens of BALB/c mice inoculated i.v. with 2.0×10^6 amastigotes of L. donovani on day 0 and treated with 1.0×10^6 viable M. bovis (BCG) as indicated. Symbols: ○, Untreated; ▲, BCG i.v. 14 days prior to and the same day of protozoan challenge; ■, BCG i.v. 30 and 14 days prior to challenge; ▬ ▪ ▬ ▪ ▬, patency level.*

inoculated i.v. with 2.0×10^6 amastigotes on day 0. Animals in groups C and D were challenged i.v. on day 0 with 2.0×10^5 amastigotes. Mice in groups B and D were inoculated i.v. with 1.0×10^7 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 2.0×10^6 amastigotes had mean spleen parasite burdens of approximately 8.5×10^6 at 30 days p.i. (Fig. 3). This parasite burden was maintained over the next 30 days. Animals given 1-log-fewer parasites (2.0×10^5) exhibited mean spleen parasite burdens of approximately 9.0×10^5 at 30 days p.i. This parasite burden gradually increased to 4.0×10^6 by 60 days p.i. Administration of 1.0×10^7 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 2.0×10^6 amastigotes had liver parasite burdens of approximately 2.4×10^8 30 days p.i. The parasite burdens steadily declined to 4.3×10^7 by 60 days p.i. Administering BCG i.v. 14 days prior to and on the day of challenge (2.0×10^6 amastigotes) significantly reduced the parasite burdens ($P < 0.01$). Three of the seven mice that had been given BCG and challenged with 2.0×10^6 amastigotes had reduced parasite burdens of 3.7×10^6. The remaining four mice had parasite burdens that were too low to detect. All mice that had been treated with BCG and challenged with 2.0×10^6 amastigotes had no detectable liver parasites 45 and 60 days p.i. Animals inoculated i.v. with 1-log-fewer amastigotes (2.0×10^5) had liver parasite burdens of 1.3×10^7 at 30 days p.i. The parasite burdens declined to a mean value to 3.5×10^6 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 2.0×10^5 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-

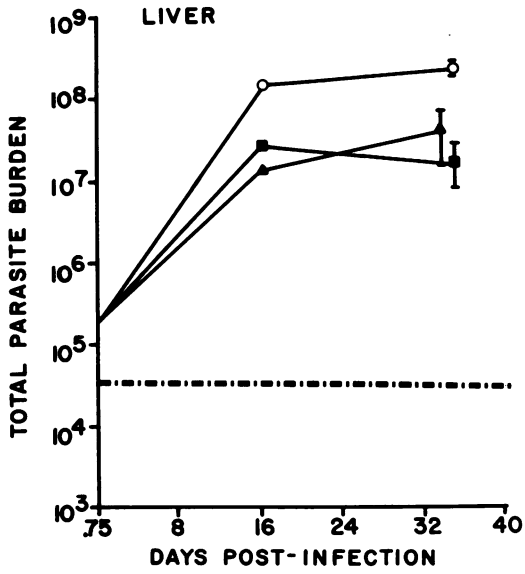

FIG. 2. *Mean total parasite burden in livers of BALB/c mice inoculated i.v. with 2.0×10^6 amastigotes of L. donovani on day 0 and treated with 1.0×10^6 viable M. bovis (BCG) as indicated. Symbols: ○, Untreated; ▲, BCG i.v. 14 days prior to and the same day of protozoan challenge; ■, BCG i.v. 30 and 14 days prior to challenge; ▬ ▪ ▬ ▪ ▬, patency level.*

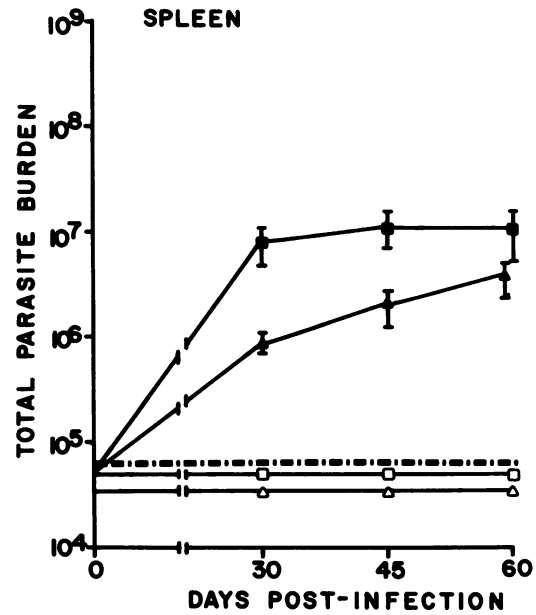

FIG. 3. *Mean total parasite burden in spleens of BALB/c mice inoculated i.v. with various doses of amastigotes of L. donovani and treated i.v. with 1.0×10^7 viable M. bovis (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols: ■, 2.0×10^6 amastigotes on day 0; □, BCG plus 2.0×10^6 amastigotes on day 0; ▲, 2.0×10^5 amastigotes on day 0; △, BCG plus 2.0×10^5 amastigotes on day 0; ▬ ▪ ▬ ▪ ▬, patency level.*

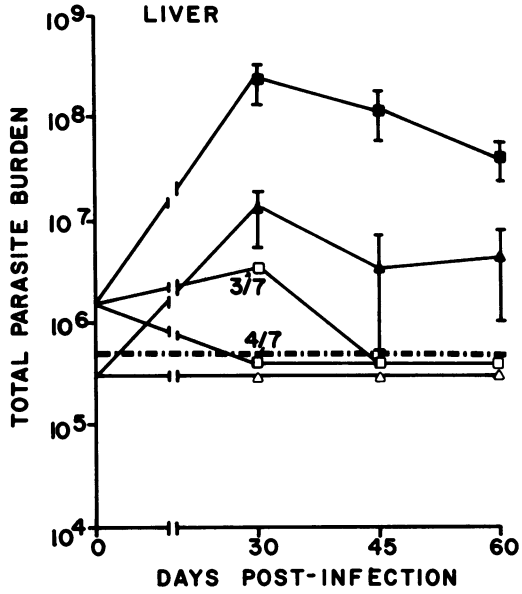

FIG. 4. *Mean total parasite burden in livers of BALB/c mice inoculated i.v. with various doses of amastigotes of L. donovani and treated i.v. with 1.0 × 10^7 viable M. bovis (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols: ■, 2.0 × 10^6 amastigotes on day 0; □, BCG plus 2.0 × 10^6 amastigotes on day 0; ▲, 2.0 × 10^5 amastigotes on day 0; △, BCG plus 2.0 × 10^5 amastigotes on day 0; ■ ■ ■ ■, patency level.*

vided into four groups of 21 mice each. Mice in groups A and B were inoculated i.v. with 2.0 × 10^6 amastigotes. Groups C and D were similarly challenged with 2.0 × 10^5 amastigotes. Groups B and D were treated i.v. with 1.0 × 10^7 VU of BCG 15 and 31 days p.i. Groups A and C were similarly treated with sterile Dubos broth. Seven mice from each group were necropsied at 30, 45, and 60 days p.i.

Mice challenged i.v. with 2.0 × 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 × 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 × 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 splenic parasites. At 30 and 60 days p.i., the spleen parasite burden of the treated group was prepatent. At 45 days p.i., four of these seven 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 signficantly 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 × 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 × 10^6 amastigotes. Seven mice from

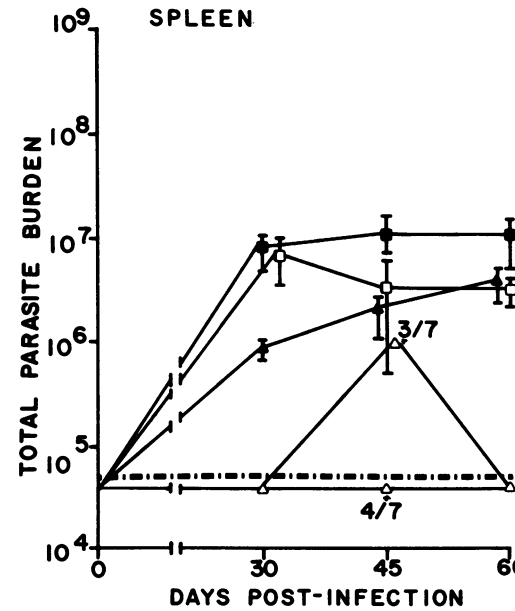

FIG. 5. *Mean total parasite burden of spleens of BALB/c mice inoculated i.v. with varying doses of amastigotes of L. donovani and treated i.v. with 1.0 × 10^7 viable M. bovis (BCG) as indicated. Symbols: ■, 2.0 × 10^6 amastigotes on day 0; □, 2.0 × 10^6 amastigotes on day 0 plus BCG 15 and 31 days p.i.; ▲, 2.0 × 10^5 amastigotes on day 0; △, 2.0 × 10^5 amastigotes on day 0 plus BCG 15 and 31 days p.i.; ■ ■ ■ ■, patency level.*

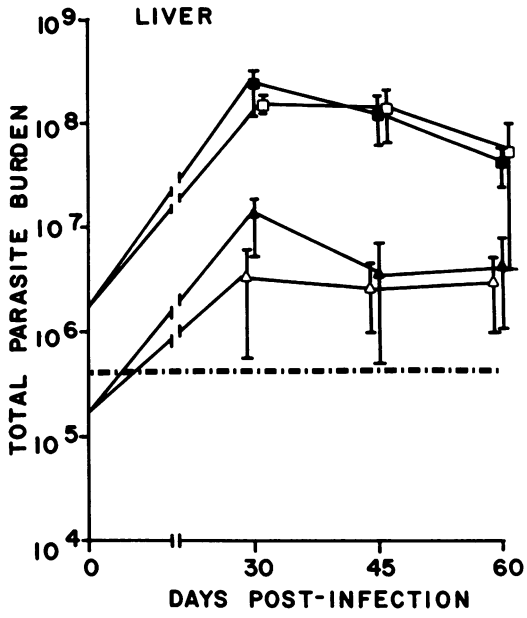

FIG. 6. *Mean total parasite burden of livers of BALB/c mice inoculated i.v. with varying doses of amastigotes of L. donovani and treated i.v. with 1.0 × 10^7 viable M. bovis (BCG) as indicated. Symbols: ■, 2.0 × 10^6 amastigotes on day 0; □, 2.0 × 10^6 amastigotes on day 0 plus BCG 15 and 31 days p.i.; ▲, 2.0 × 10^5 amastigotes on day 0; △, 2.0 × 10^5 amastigotes on day 0 plus BCG 15 and 31 days p.i.; ■ ■ ■ ■, patency level.*

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 × 10^6 amastigotes of *L. donovani*. Nontreated mice attained spleen parasite burdens of 8.5 × 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 × 10^6 amastigotes. Infected mice reached a peak liver parasite burden of 2.4 × 10^8 amastigotes at 30 days p.i. The liver parasite burdens declined steadily to 4.3 × 10^7 by 60 days p.i. Mice inoculated i.p. with BCG and challenged i.v. with 2.0 × 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 × 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 *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 *Mycobacterium phlei*, BCG, *Mycobacterium butyricum*, or two atypical isolates of mycobacteria from man supported significantly lower populations of *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 *L. donovani* resisted challenge with *Mycobacterium tuberculosis*.

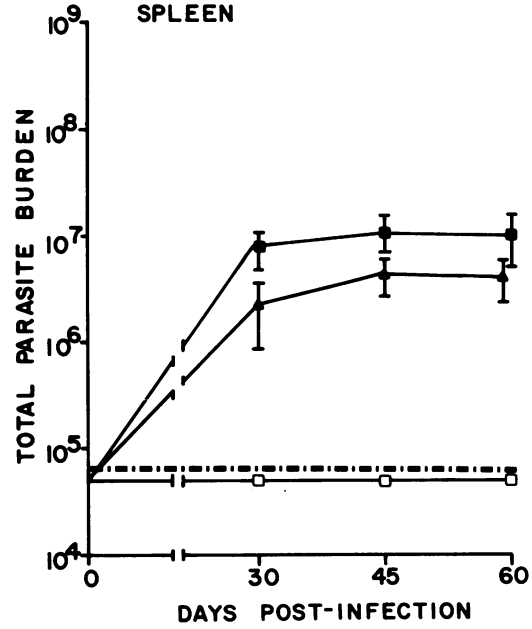

FIG. 7. *Mean total parasite burden in spleens of BALB/c mice inoculated i.v. with 2.0 × 10^6 amastigotes of L. donovani and treated by various routes with 1.0 × 10^7 viable M. bovis (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols: ■, Amastigotes on day 0; □, amastigotes on day 0 plus BCG i.v.; ▲, amastigotes on day 0 plus BCG i.p.; ■ ■ ■ ■, patency level.*

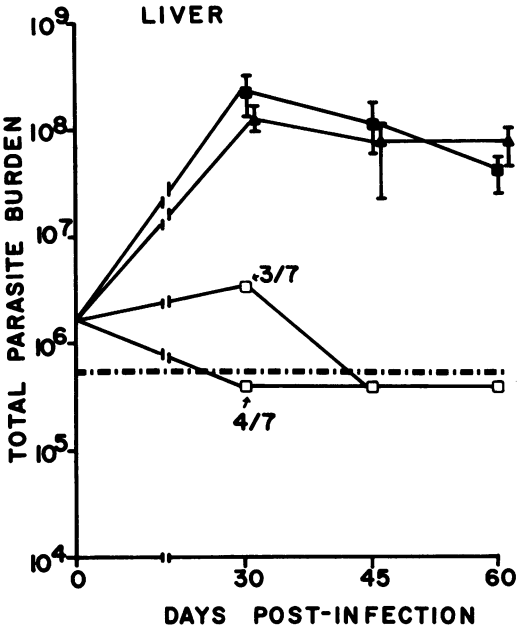

FIG. 8. *Mean total parasite burden in livers of BALB/c mice inoculated i.v. with 2.0 × 10^6 amastigotes of L. donovani and treated by various routes with 1.0 × 10^7 viable M. bovis (BCG) 14 days prior to and on the same day of protozoan challenge. Symbols: ■, Amastigotes on day 0; □, amastigotes on day 0 plus BCG i.v.; ▲, amastigotes on day 0 plus BCG i.p.; ■ ■ ■ ■ ■, patency level.*

The effect of prior treatment with BCG on visceral leishmaniasis in BALB/c mice is dependent on several factors: (i) dose of BCG, (ii) number of challenge parasites, (iii) route of treatment, and (iv) time of treatment with BCG in relation to parasite challenge.

The data from the first experiment demonstrate the effect of the time of administration of BCG upon the parasite burdens in mice infected with *L. donovani*. Mice inoculated with BCG either 30 and 14 days p.i. or 14 days prior to and on the same day of infection with the protozoa had significantly lower liver parasite burdens compared to the control mice. However, those animals inoculated with BCG 14 days prior to and on the day of infection with *L. donovani* did not develop spleen parasite burdens that were capable of being detected by present methods, whereas those inoculated with BCG 30 and 14 days prior to infection had high spleen parasite burdens at 16 days, but these were effectively reduced by day 32. A possible explanation of these results is that the second dose of BCG caused a rapid release of soluble mediators that were capable of producing a profound effect upon both lymphocytes 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 spleen 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 × 10^7 rather than with 1.0 × 10^6 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 × 10^6 or 2.0 × 10^5 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-

trated. 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 1.0×10^5 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 parasitized 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.

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LITERATURE CITED

1. **Adler, S.** 1965. Immunology of leishmaniasis. Isr. J. Med. Sci. 1:9–13.
2. **Allwood, G. G., and G. L. Asherson.** 1972. Depression of delayed hypersensitivity by pretreatment with Freund-type adjuvant. III. Depressed arrival of lymphoid cells at recently immunized lymph nodes in mice pretreated with adjuvant. Clin. Exp. Immunol. 11:579–584.
3. **Anacker, R. L., J. Matsumoto, E. Ribi, R. F. Smith, and K. Yamamoto.** 1973. Enhancement of resistance of mice to tuberculosis by purified components of mycobacterial lipid fractions. J. Infect. Dis. 127:357–364.
4. **Biozzi, B., B. Benaceraff, F. Grumbach, B. N. Halpern, J. Levadite, and N. Rist.** 1954. Etude de l'activate granulo pexique du système reticuloendothélial au cours de l'infection tuberculouse expérimentale de la souris. Ann. Inst. Pasteur Paris 87:291–300.
5. **Biozzi, B., B. N. Halpern, and C. Stiffel.** 1963. Stimulation du systeme réticulo-endothéthial (S.R.E.) par l'extrait microbien wxb 3148 et résistance aux infections expérimentales, p. 205–220. *In* M. Bernard and N. Halpern (ed.), Colloques Internationaux du Centre National de la Recherche Scientifique no. 115. Centre National de la Recherche Scientifique, Paris.
6. **Blanden, R. V.** 1968. Modification of macrophage function. J. Reticuloendothel. Soc. 5:179–202.
7. **Blewett, T. M., D. M. H. Kadivar, and E. J. L. Soulsby.** 1971. Cutaneous leishmaniasis in the guinea pig. Delayed-type hypersensitivity, lymphocyte stimulation, and inhibition of macrophage migration. Am. J. Trop. Med. Hyg. 20:546–551.
8. **Bloom, B. R., and B. Bennett.** 1968. Migration inhibitory factor associated with delayed-type hypersensitivity. Fed. Proc. 27:13–16.
9. **Bryceson, A. D. M.** 1970. Immunological aspects of clinical leishmaniasis. Proc. R. Soc. Med. 63:1056.
10. **Bryceson, A. D. M.** 1970. II. Pathogenesis of diffuse cutaneous leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 64:387–393.
11. **Bryceson, A. D. M., R. S. Bray, and D. C. Dumonde.** 1974. Experimental cutaneous leishmaniasis. IV. Selective suppression of cell-mediated immunity during the response of guinea pigs to infection with *Leishmania enriettii*. Clin. Exp. Immunol. 16:189–202.
12. **Bryceson, A. D. M., P. M. Preston, R. S. Bray, and D. C. Dumonde.** 1972. Experimental cutaneous leishmaniasis. II. Effects of immunosuppression and antigenic competition on the course of infection with *Leishmania enriettii* in the guinea pig. Clin. Exp. Immunol. 10:305–335.
13. **Bryceson, A. D. M., and J. L. Turk,** 1971. The effect of prolonged treatment with ALS on the course of infections with BCG and *Leishmania enriettii* in the guinea pig. J. Pathol. 104:153–165.
14. **Clinton, B. A., L. A. Stauber, and N. C. Palczuk.** 1969. *Leishmania donovani*: antibody response to chicken ovalbumin by infected golden hamsters. Exp. Parasitol. 25:171–180.
15. **David, J. R., H. S. Lawrence, and L. Thomas.** 1964. Delayed hypersensitivity *in vitro*. II. Effect of sensitized cells on normal cells in the presence of antigen. J. Immunol. 93:274.
16. **DeAlencar, J. E., A. Ilardi, and S. Panpiglione.** 1966. The complement-fixation reaction in the diagnosis of visceral leishmaniasis; antigen from acid alcohol resistant bacteria. Parasitologia 8:147–181.
17. **Evans, R., and P. Alexander.** 1970. Cooperation of immune lymphoid cells with macrophages in tumor immunity. Nature (London) 228:620–622.
18. **Guirges, S. Y.** 1971. Natural and experimental reinfection of man with Oriental sore. Ann. Trop. Med. Parasitol. 65:197–205.
19. **Hanna, M. G., B. Zbar, and H. J. Rapp.** 1972. Histopathology of tumor regression after intralesional injection of *Mycobacterium bovis*. I. Tumor growth and metastasis. J. Natl. Cancer Inst. 48:1441–1449.
20. **Heyneman, D.** 1971. Immunology of *Leishmania*. Bull. W.H.O. 44:499–514.
21. **Hibbs, J. B., L. H. Lambert, and J. S. Remington.** 1972. Adjuvant induced resistance to tumor development in mice. Proc. Soc. Exp. Biol. Med. 139:1053–1056.
22. **Hibbs, J. B., L. H. Lambert, and J. S. Remington.** 1972. Control of carcinogenesis: a possible role for the activated macrophage. Science 177:998–1000.
23. **Hibbs, J. B., L. H. Lambert, and J. S. Remington.** 1972. *In vitro* nonimmunologic destruction of cells with abnormal growth characteristics by adjuvant activated macrophages. Proc. Soc. Exp. Biol. Med. 139:1049–1052.
24. **Hibbs, J. B., L. H. Lambert, and J. S. Remington.** 1972. Possible role of macrophage mediated nonspecific cytotoxicity in tumor resistance. Nature (London) New Biol. 235:48–50.
25. **Khaleque, K. A.** 1962. A new method for preparing an antigen from Kedrowsky's bacillus for the complement fixation test for kala-azar. J. Pathol. Bactiol. 83:284–287.
26. **Khaleque, K. A.** 1965. Complement fixation test for kala-azar with an antigen prepared from acid fast bacillus. Pak. J. Med. Res. 4:234–240.
27. **Kojevnikov, P. V.** 1961. Some results of the works of Soviet scholars in the study of cutaneous leishmaniasis. Dermatologica (Basel) 123:341–356.
28. **Krahenbuhl, J. L., and J. S. Remington.** 1971. *In vitro* induction of nonspecific resistance in macrophages by specifically sensitized lymphocytes. Infect. Immun. 4:337–343.
29. **Lainson, R., and R. S. Bray.** 1966. Studies on the immunology and serology of leishmaniasis. II. Cross-immunity experiments among different forms of American cutaneous leishmaniasis in monkeys. Trans. R. Soc. Trop. Med. Hyg. 60:526–532.
30. **Larson, C. L., R. E. Baker, R. N. Ushijima, M. B. Baker, and C. Gillespie.** 1972. Immunotherapy of Friend Disease in mice employing viable BCG vaccine (36534). Proc. Soc. Exp. Biol. Med. 140:700–702.
31. **Larson, C. L., R. N. Ushijima, J. J. Florey, R. E. Baker, and M. B. Baker.** 1971. Effect of BCG on

Friend Disease virus in mice. Nature (London) New Biol. 229:243-244.
32. **Lodmell, D. L., A. Niwa, K. Hayashi, and A. L. Notkins.** 1973. Prevention of cell-to-cell spread of herpes simplex virus by leukocytes. J. Exp. Med. 137:706-720.
33. **Mackaness, G. B., and R. B. Blanden.** 1967. Cellular immunity. Prog. Allergy 11:89-140.
34. **Majumdar, T. D.** 1967. Post-kala-azar dermal leishmaniasis. Dermatol. Int. 6:174-177.
35. **Manson-Bahr, P. E.** 1964. Variations in the clinical manifestations of leishmaniasis caused by *Leishmania tropica*. J. Trop. Med. Hyg. 67:85-87.
36. **Neal, R. A., P. C. C. Garnham, and S. Cohen.** 1969. Immunization against protozoal diseases. Br. Med. Bull. 25:195-201.
37. **North, R. J.** 1969. Cellular kinetics associated with the development of acquired cellular resistance. J. Exp. Med. 130:299-311.
38. **Nussenzweig, V.** 1957. Complement-fixation test for visceral leishmaniasis with *Mycobacterium tuberculosis* antigen. Technique, sensitivity, and specificity. Hospital (Rio do Janeiro) 51:217-226.
39. **Sagher, F., S. Verbi, and A. Zuckerman.** 1965. Immunity to reinfection following recovery from cutaneous leishmaniasis (Oriental sore). J. Invest. Dermatol. 24:417.
40. **Stauber, L. A.** 1955. Leishmaniasis in the hamster, p. 76-90. *In* W. H. Cole (ed.), Some physiological aspects and consequences of parasitism. Rutgers University Press, New Brunswick, N.J.
41. **Stauber, L. A.** 1958. Host resistance to the Khartoum strain of *L. donovani*. Rice Inst. Pam. 45:80-96.
42. **Torrealba, J. W., and J. Chaves-Torrealba.** 1964. BCG antigen used in the complement fixation test for the diagnosis of kala-azar. Rev. Inst. Med. Trop. Sao Paulo 6:252-253.
43. **Tremonti, L., and B. C. Walton.** 1970. Blast transformation and migration-inhibition in toxoplasmosis and leishmaniasis. Am. J. Trop. Med. 19:49-56.