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Growth of *Mycobacterium marinum* in the Footpads of T-Cell-Depleted Mice

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Mycobacterium marinum strains 1218 and 1219 were inoculated into the hind footpads of T-cell-depleted specific pathogen-free C57B1/6 mice, and the growth and survival of the organisms at the site of injection, the draining popliteal lymph node, and the spleen and lung were quantitated for up to 70 days. T-cell depletion largely ablated the normal cell-mediated antituberculous response to the *M. marinum* population. The mice were able to control the further growth of the inoculum within the footpad only after it had reached 5 to 10 times that present in the normal controls. The high temperature-adapted strain (37 C; strain no. 1218) induced an increasing infection in the liver, spleen, and lungs of the THXB mice, and the infection eventually spread to the opposite footpad and to the tail skin. Strain 1219 gave rise to considerable systemic involvement in the THXB host despite its inability to survive at 37 C, but the size of the splenic and lung populations was considerably lower than in the 1218-infected animals. Both *M. marinum* infections persisted in the tissues of the T-cell-depleted mice with no indication of a cell-mediated immune response. Footpad swelling in the *M. marinum*-infected mice was not greatly reduced by T-cell depletion, and, if anything, tended to persist at high levels long after the swelling of the control feet had gone into a decline. On the other hand, incorporation of tritiated thymidine by cells within the infected footpads, the draining lymph node, and the spleen was considerably reduced in the T-cell-depleted host compared with control values. Late in the infection, there was a significant increase in the amount of label taken up by the cells in the footpads of the T-cell-depleted host.

Inoculation of *Mycobacterium marinum* into the footpads of normal mice is followed by a period of rapid growth at the site of inoculation but with relatively little systemic involvement (6, 11). However, strains of *M. marinum* which have been adapted in the laboratory to also grow at 37 C were able to induce a much higher degree of systemic infection, and this was ascribed to their ability to multiply and survive at normal body temperatures. The generalized systemic infection brought about by these high temperature-adapted strains induced higher levels of cell-mediated immunity against a subsequent challenge population compared to that seen when the mice were "vaccinated" with strains unable to multiply or survive at 37 C.

M. marinum multiplies extensively in situ when introduced into the normal mouse footpad, often reaching maximum counts of 10^7 viable bacilli in 7 to 10 days. The viable population then declines again under the influence of an emerging cell-mediated immune response. Rees (15) showed that prior T-cell depletion increased the yield of *M. leprae* which accumulated within an infected mouse footpad by as much as 100-fold, presumably by depressing the normal cell-mediated host immune responses. The present study indicates that T-cell depletion also affects the expression of footpad immunity to an *M. marinum* infection in the footpad, increasing both the maximum yield and the persistence of the bacterial population in vivo.

MATERIALS AND METHODS

Animals. Four-week-old specific pathogen-free C57B1/6 female mice were obtained from Jackson Laboratories, Bar Harbor, Me., and maintained as described elsewhere (1). Half of the mice were thymectomized surgically, exposed to 900 R of whole body irradiation 7 days later, and immediately reconstituted with 2×10^6 normal syngeneic bone marrow cells (12). The mice were given tetracycline-enriched water for 4 weeks and removed from the drug 7 days prior to infection. Sham thymectomized controls were always included together with normal untreated mice.

Organisms. *M. marinum* (TMC no. 1218 and 1219) as well as BCG Montreal (TMC no. 1012) were

obtained from the Trudeau Mycobacterial Culture Bank, Saranac Lake, N.Y. *M. marinum* strains were grown in modified Sauton liquid medium at 32 C with moderate agitation (7). BCG Montreal suspensions were prepared (4) and stored at −70 C as described earlier (9). Inocula were prepared from the frozen suspensions as described elsewhere (2). Mice were infected with approximately 10^6 viable bacilli injected into a hind footpad in a volume of 0.02 ml of saline. Enumeration of the viable populations within the two hind footpads, the draining lymph nodes, and the lung and spleen was made using saline homogenates of the separate organs as described elsewhere (5). Suitable saline dilutions were spotted onto Middlebrook 7H10 agar plates which were incubated at the optimum growth temperature for the organism in sealed plastic bags for 2 to 4 weeks before counting.

Cellular proliferation in the host tissues. Proliferation by cells within the footpad, the draining lymph node, and the spleen was measured by pulse labeling the mice with 20 μCi of tritiated thymidine ([^{3}H]TdR) 30 min prior to sacrifice (12). The whole foot and the popliteal lymph node, as well as known proportions of the spleen, were homogenized in trichloroacetic acid, and the ^{3}H-labeled deoxyribonucleic acid content was determined using a Beckman LS-100 liquid scintillation spectrometer (13).

Foot swelling. The increase in footpad thickness was measured at intervals after infection using a Schnelltaster dial gauge calipers (4). An increase of 1.8 U (0.18 mm) or more was considered to be significant at the 1% level.

RESULTS

Growth of M. marinum 1218 in THXB mice. *M. marinum* is a temperature-adapted strain capable of growing at 37 C although its optimal growth temperature is approximately 34 C (6). Inoculation of about 10^6 viable bacilli into the footpads of 50 THXB, XB, and normal C57B1 mice yielded the growth curves shown in Fig. 1. The footpad population within the right hind foot increased about 50-fold in the THXB mice, reaching a maximum of 5×10^7 viable bacilli per pad by day 14 (Fig. 1). There was little indication of any immune response by the T-cell-depleted host, and the viable counts remained in the region of 10^7 bacilli per footpad until at least day 60, when the study had to be concluded for lack of animals. The right popliteal lymph node counts rose sharply to about 5×10^5 bacilli by day 10 and remained at this level for most of the experiment, with no sign of a subsequent immune decline. The infection quickly spread to the lung, liver, and spleen, with steadily increasing counts in all three test organs (Fig. 1). Viable bacilli could also be recovered from the opposite (uninoculated) footpad beginning about day 20 with a considerable amount of growth occurring within the pad itself, followed by some spread to the left

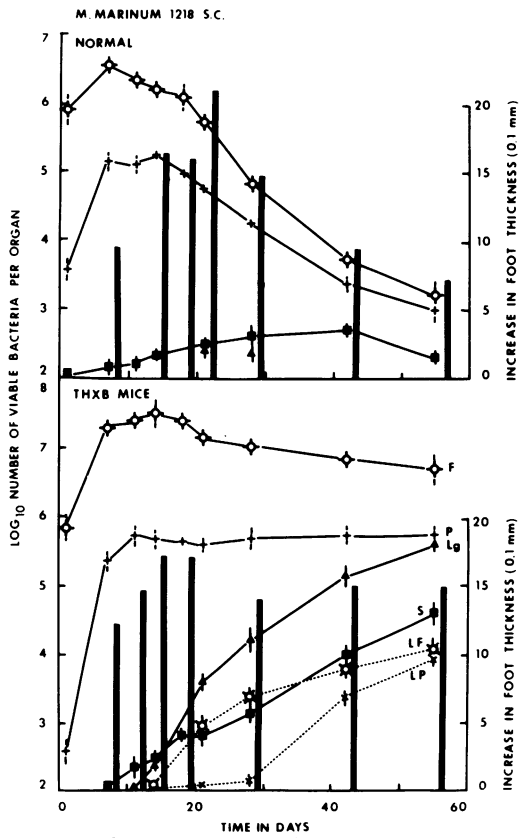

FIG. 1. *Growth of M. marinum 1218 in the footpads (F), popliteal lymph node (P), spleen (S), lung (▲), left footpad (LF), and left popliteal lymph node (LP) in normal C57B1 mice (top) or THXB mice (bottom) following inoculation of 10^6 viable bacilli into the right hind footpad. The histograms represent the increase in the thickness of the injected foot at increasing time intervals.*

popliteal lymph node about day 56. The right foot developed a substantial amount of swelling starting 2 or 3 days after inoculation (Fig. 1), reaching a maximum on day 15. There was no indication of a later decline in swelling, which remained at about a 100% increase in overall foot thickness over most of the study.

Both the XB and normal controls gave essentially identical growth curves, and only the latter data have been included in this paper. The footpad population in the control mice increased only twofold over the first 10 days, followed by a relatively rapid 100-fold decline over the next 40 to 50 days. The infection spread to the draining popliteal lymph node, as well as to the liver and spleen, but the amount of systemic involvement was generally less than it had been in the T-cell-depleted mice, with a slower rate of growth in both the liver and

spleen. The growth curves for the normal host infected with strain 1218 were very similar to those reported earlier using CD-1 mice (6). It should be noted that the lungs of these mice were minimally infected during the early stages of growth, but that these counts later declined rapidly towards zero. The normal controls showed a 150% increase in foot thickness (day 22), and although this swelling declined slowly, significant readings (3.6 ± 0.2 U) were still observable after 100 days. At this time, the bacterial counts for these footpads were less than 100 viable bacilli.

Growth of M. marinum strain 1219 in THXB mice. *M. marinum* strain 1219 introduced into the footpads of T-cell-depleted mice showed a 10-fold increase in viable counts over the first 14-day period. The viable population then remained at about this level throughout the remainder of the study (Fig. 2). The popliteal lymph node counts also increased nearly 100-fold, and then they too remained steady until at least day 70. The lungs and the spleens of the T-cell-depleted mice developed small though significant counts with nearly 1,000 viable bacilli still present after 70 days. Viable counts carried out on homogenates of the left foot and the left popliteal lymph node were extremely variable, both within a single group

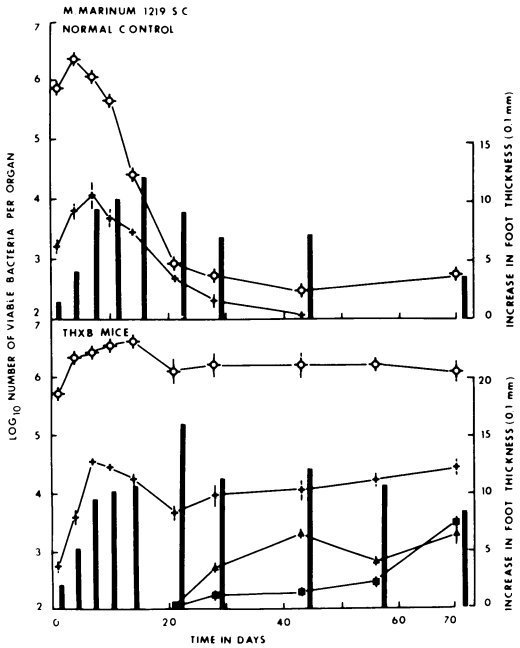

FIG. 2. *Growth of M. marinum 1219 following the footpad inoculation of normal C57Bl mice (top) or THXB mice (bottom). See legend to Fig. 1 for further details.*

and at different time points, and it was not possible to quantitate them accurately. Large skin ulcers were observed to develop on the tails and ears of some of the THXB mice as the infection progressed. There was no indication of an immune response to the *M. marinum* population in the infected footpad or its draining lymph node. The increase in footpad thickness observed in these mice was somewhat lower than in the 1218-infected animals, varying about a peak of 100% seen on day 20 (Fig. 2).

Infection of the sham-thymectomized and normal control mice with 10^6 viable *M. marinum* 1219 resulted in a short initial period of growth followed by a rapid decline in viability, so that the counts on day 30 represented only 0.01% of the earlier maximum (Fig. 2). Although the draining popliteal lymph node became heavily infected by day 10, the viable counts for this organ also quickly declined to near undetectable levels. Neither the spleen, the lungs, nor the opposite footpad contained detectable numbers of bacilli at any time during the experiment. The amount of footpad swelling seen in the 1219 controls was generally smaller than had been observed with strain 1218, despite the fact that the viable counts obtained in the footpads of the two groups of mice were very similar (compare Fig. 1 and 2).

The behaviour of the 1218 infection in the footpad more closely resembled that shown earlier for BCG Montreal (5) than it did the 1219 strain of *M. marinum*. BCG Montreal did not multiply greatly when introduced into the footpads of T-cell-depleted mice (Fig. 3), although the established infection did persist in vivo with little change over the next 60 days. The infection quickly spread to the draining lymph node and to the spleen. After the early growth phase, the node, lung, and spleen populations passed into a prolonged stationary phase with no indication of a developing local immune response. The infection did not spread to the left footpad or its draining lymph node, and there was no significant increase in footpad thickness at any time despite the substantial population of viable mycobacteria within the right hind foot.

Immune response by M. marinum-infected THXB mice. Substantial cross-protection has been demonstrated between *M. marinum* and *M. tuberculosis* in doubly infected mice (6). By analogy with earlier studies with BCG-infected T-cell-depleted mice (9a), it was thought unlikely that the *M. marinum* animals would be able to mount an effective immune response against a *M. tuberculosis* Erdman challenge. The growth curves for the spleen and lung Erd-

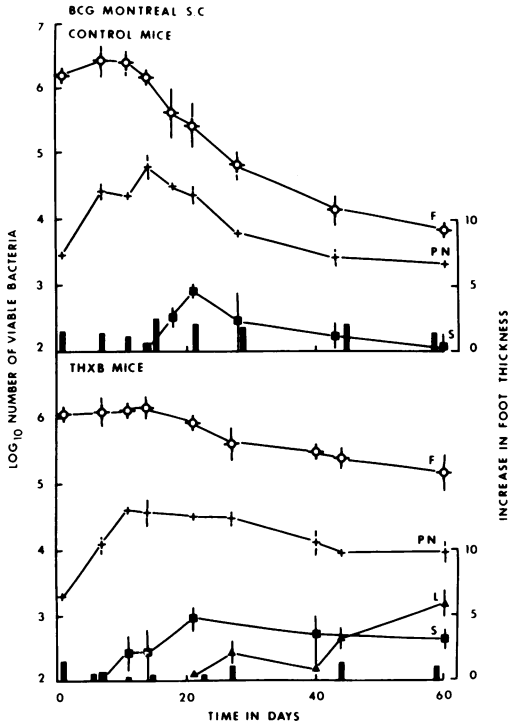

FIG. 3. *Growth of BCG Montreal following the footpad inoculation of normal C57B1 mice (top) or THXB mice (bottom). See legend to Fig. 1 for further details.*

man populations in the *M. marinum* "vaccinated" THXB mice were virtually identical with those seen in the unvaccinated controls (Fig. 4), indicating a complete lack of cell-mediated immunity in the T-cell depleted host.

Cellular proliferation in M. marinum-infected THXB mice. Incorporation of [^{3}H]TdR by cells within the *M. marinum*-infected footpad increased two- to fivefold over the first 10 days of the experiment (Fig. 5) and then declined rapidly to near normal values by day 21. Over the same time interval, the bacterial population within the pad showed very little change in viability, while the footpad more than doubled in thickness. A similar though smaller peak in incorporation was also seen in the draining popliteal lymph node. On the other hand, the rate of splenic incorporation increased only slowly up to day 14 and then declined again sharply. This minimal value was followed by a slower increase in the [^{3}H]TdR uptake by all three test organs up until the completion of the study on day 42 (Fig. 5). There was also a late rise in [^{3}H]TdR incorporation by the opposite footpad, although very few viable bacilli were present in that tissue at the time.

Incorporation data for strain 1218-infected T-cell-depleted mice revealed two interesting variations from the above picture. There was little or no decline in the early peak uptake over the day 10 through 21 period; rather, incorporation of label occurred at a relatively constant level throughout the experiment, although there was a small peak about day 30 (Fig. 5). Incorporation by cells within the infected popliteal lymph node was largely ablated in the THXB host, and (although not shown in Fig. 5) the lung values remained at about 5,000 counts/min throughout the experiment, despite the fact that as many as 10^5 viable *M. marinum* 1218 could be recovered from this organ by day 50 (Fig. 1).

Control mice infected with *M. marinum* 1219 also showed a peak in footpad incorporation

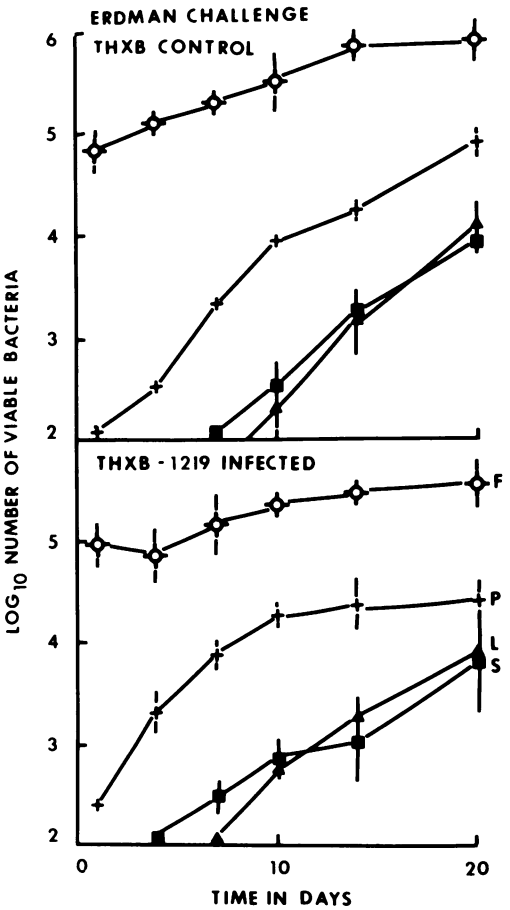

FIG. 4. *Growth of M. tuberculosis Erdman in the THXB mice (top) or THXB mice infected 50 days previously with M. marinum 1219. Both groups of mice received 10^5 viable Erdman injected into the left hind footpad.*

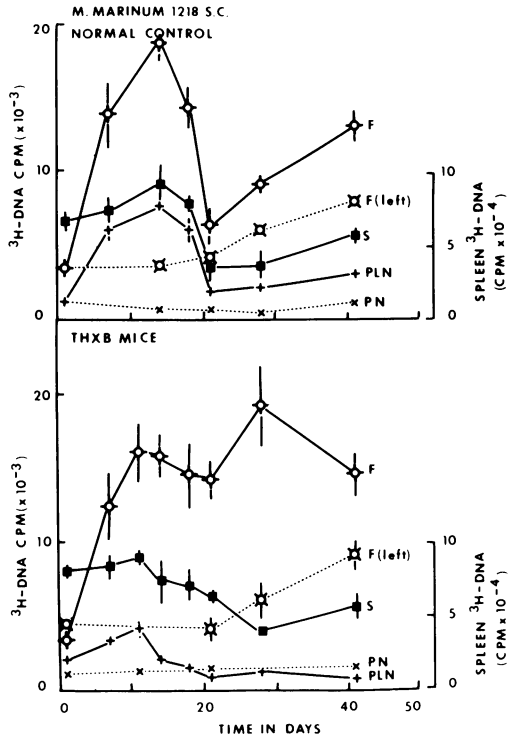

FIG. 5. *Incorporation of [^{3}H]TdR by cells in the footpads (F), popliteal lymph nodes (PLN), and spleens (S) of normal (top) and THXB (bottom) mice infected in the right hind footpad with 10^6 M. marinum 1218. PN represents the left (uninfected) popliteal lymph node.*

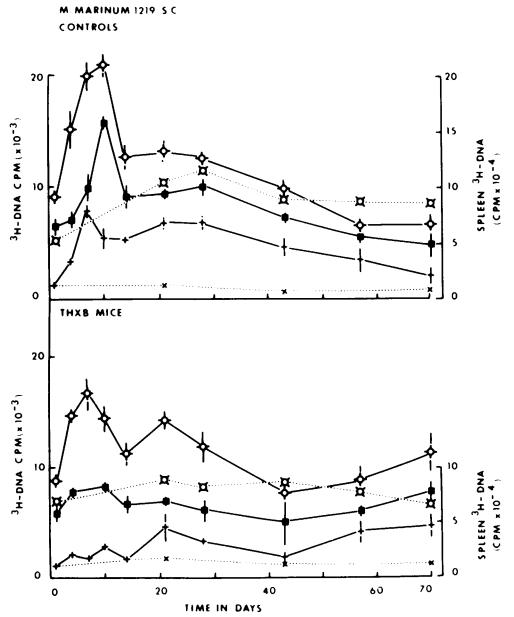

FIG. 6. *Incorporation curves for normal (top) and THXB (bottom) mice infected in the right hind footpad with 10^6 M. marinum 1219. See legend to Fig. 5 for further details.*

about day 10 (Fig. 6), followed by a rapid drop to near normal levels by day 14, but with no later peak in uptake equivalent to that seen in the 1218 experiment. There was a slow increase in [^{3}H]TdR uptake in the contralateral footpad over the first 30 days despite the absence of detectable bacilli within the pad (Fig. 2). The splenic curve showed a sharp increase in ^{3}H-labeled deoxyribonucleic acid about day 10, followed by an equally rapid decline, and this correlated with the behavior of the footpad and draining node curves. The splenic ^{3}H-labeled deoxyribonucleic acid curve did not mirror the increasing bacterial counts seen within that organ as the infection progressed (Fig. 2). The 1219-infected THXB mice generally showed a considerable reduction in the rate of uptake of [^{3}H]TdR by all test organs compared with the controls. The footpad uptake increased over the first 20 days or so of the infection followed by a slow decline to near normal values (Fig. 6). There was no change in splenic or popliteal lymph node incorporation rates during the early part of the infection, although later a slow rise was seen in the lymph node curve.

DISCUSSION

Mycobacterial growth within the mouse footpad usually ceases once the bacterial population reaches a maximum of approximately 10^6 viable bacilli at the site of inoculation (5, 10). When larger numbers of viable mycobacteria are inoculated into the pad, the viable counts usually decline until they reach a level of about 10^6 bacilli. Injection of 10^6 *M. leprae* into the normal mouse footpad is followed by an approximately 90% mortality over the first few weeks of the infection (14). On the other hand, injection of 5×10^3 viable bacilli is followed by a considerable period of growth which only ceases as the bacterial population approaches 10^6 bacilli per pad. Prior T-cell depletion of the host increases the "plateau" population 10 to 100 times, but growth of the organisms was eventually limited even in the immunosuppressed animal. This limitation did not seem to be immunologically mediated since the "vaccinated" T-cell-depleted animals were unable to control the growth of a subsequent challenge infection (Fig. 4). This is consistent with earlier data reported for BCG-infected animals (3, 11). The enigma of a mechanism able to limit the growth of a footpad inoculum of *M. marinum* in

the THXB mouse in the face of a total inability to express an effective acquired antituberculous resistance to rechallenge is puzzling. One explanation could be a limitation of the original footpad population by nutritional or physicochemical factors imposed on the bacterial population by the limited space within the footpad.

Injection of *M. marinum* strain 1219 into the THXB mouse footpad resulted in an infection somewhat resembling that reported for *M. leprae* (16, 17). However, in addition to the fact that there was an extensive systemic involvement of the lymphoreticular system of the T-cell-depleted host infected with *M. marinum* (Fig. 1), the footpad infection of both normal and THXB mice results in a severe inflammatory response leading to severe tissue toxicity, necrosis, and even ulceration of the footpad (8, 11). No such local response is seen when *M. leprae* is introduced into the footpad, even in the immunosuppressed host (16). The reason for the spectacular increase in foot thickness seen in both T-cell-depleted and normal mice is still not clear. The response is similar to that seen when lipid irritants such as castor oil are introduced into the footpad (N. E. Morrison, unpublished data), and this effect can also be induced with dead mycobacterial suspensions (6). This tissue response is not affected by prior T-cell depletion and the cellular elements of the response (mainly polymorphonuclear leukocytes) are quite different from those seen in the corresponding *M. leprae* lesions (10, 14). The former seem to resemble the type of chronic skin abscesses seen when an extracellular parasite is introduced into the tissues (C. C. Shepard, personal communication).

M. marinum has been suggested as a model for *M. leprae* (11, 17) mainly because of its low temperature growth optimum (32 to 34 C), but its very rapid mean generation time, inflammatory response, and tendency to spread systemically all appear to contraindicate its further use in this regard unless some unexpectedly close antigenic relationship is found to exist between *M. marinum* and *M. leprae*. Thus, at present there is little reason to think that *M. marinum* will prove to be a suitable model for studying the host-parasite interactions developed as an inoculum of *M. leprae* establishes itself in the mouse footpad.

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