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Growth and Immunogenicity of Photochromogenic Strains of Mycobacteria in the Footpads of Normal Mice

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Specific pathogen-free CD-1 mice were infected subcutaneously in the footpad with *Mycobacterium kansasii*, three strains of *M. marinum*, and two strains of *M. simiae-habana*, and the growth of the organisms in the footpad, the draining popliteal lymph node, and the lung and spleen was followed quantitatively for up to 60 days. The ability of a footpad inoculum of *M. marinum* to spread to the lungs and spleen correlated with the ability of the organism to survive and multiply at 37 C in in vitro cultures. The amount of footpad swelling which developed in the *M. kansasii*- and *M. marinum*-infected mice varied depending upon the strain of organism and the size of the original footpad inoculum. Injection of dead *M. marinum* into the footpad also induced an extensive amount of swelling which varied with the strain used, as well as being dose dependent. *M. marinum*- and BCG-vaccinated mice were protected against a later footpad challenge with *M. marinum* or the highly mouse virulent *M. tuberculosis* strain Erdman. The significance of this finding is discussed in relation to cross-protection studies using a variety of mycobacteria in the footpad infection model.

Mycobacterium marinum is a photochromogenic mycobacterium able to induce a self-limiting localized infection within the mouse footpad (2, 8), somewhat analogous to that reported for *M. leprae* (14). As a result, *M. marinum* has been suggested as an alternative experimental model for studying the role of host immunity to *M. leprae* infections in the mouse footpad (16). Ng et al. (13) showed that small inocula of *M. marinum* injected into the footpads of normal mice multiply rapidly to a maximum of about 10^6 to 10^7 viable bacilli over an 8- to 10-day period. This was followed by a steady decline in viability over the next 2 or 3 weeks. The footpad infection also induces a severe, prolonged inflammatory response (2, 13), the lesions often being later associated with a severe ulceration (8). This local swelling at the site of inoculation has been used as an index of bacterial growth within the footpad since good agreement usually occurs between these two parameters, at least during the early stages of the infection (13). Footpad thickness measurements provide a simpler alternative to the tedious serial viable and total bacterial counts previously used to follow the development of the infection in vivo. However, data presented in the present study indicates that such footpad swelling reactions are not necessarily directly related to the number of viable bacilli in the footpad. The observed discrepancies between the degree of foot swelling and the viable counts obtained later for the corresponding organ homogenates renders the former parameter of limited value so far as studies of cross-protection in footpad challenged animals are concerned.

MATERIALS AND METHODS

Organisms. *M. kansasii* (Forbes, TMC no. 1201), *M. marinum* (TMC no. 1218 and 1219), *M. simiae* (TMC no. 5131), and *M. habana* (TMC no. 5135) were obtained from the Trudeau Mycobacterial Culture Bank, Saranac Lake, N.Y. *M. marinum* strain HT was a primary isolate from a human skin lesion which had been subcultured onto artificial culture media only once prior to its mouse inoculation. *M. tuberculosis* Erdman (TMC no. 107) and BCG Montreal (TMC no. 1012) were described in earlier papers (3). Both organisms were grown in modified Sauton medium on a magnetic stirrer for 6 to 8 days (7). *M. marinum* cultures were incubated at 32 C, whereas the other strains were routinely grown at 37 C. The liquid cultures were first diluted 1 in 10 with fresh culture media, frozen in 1-ml ampoules, and stored at −70 C (10). The viability of each suspension was checked by rapidly thawing the ampoule at 37 C, homogenizing the preparation briefly, and plating suitable saline dilutions on Middlebrook 7H10 agar (Difco). The plates were sealed in plastic bags, and the *M. marinum* cultures were incubated at 32 C for 7 to 10 days. Other cultures were incubated at 37 C for 3 to 4 weeks before counting.

Animals. Four-week-old specific pathogen-free

CD-1 mice (Charles River Farms, Wilmington, Mass.) were maintained 10 to a cage on sterile bedding under Isocaps and fed sterile vitamin-enriched pellets and chlorinated water ad lib.

The mice were infected with 10^6 viable mycobacteria by the intravenous route or with 10^4 to 10^6 organisms injected subcutaneously into the right hind footpad in a volume of 0.02 ml of saline (6). The viability of the inoculum was checked immediately following its injection by plating suitable 10-fold saline dilutions on 7H10 agar.

Challenge of infected mice with *M. marinum* or *M. tuberculosis* Erdman was carried out by footpad inoculation 50 days after the primary inoculation. The growth of the challenge organism at the site of inoculation, in the draining popliteal lymph node, and in the spleen and lung was followed over a 20-day period (6). In BCG-vaccinated controls, the residual vaccine and the *M. tuberculosis* colonies were distinguished by double plating the organ homogenates on 7H10 agar with and without 2 μg of 2-thiophene carboxylic acid hydrazide per ml (5). *M. marinum* and *M. tuberculosis* could be readily distinguished on the basis of growth differences on 7H10 agar after incubation at 32 C.

Enumeration of the in vivo bacterial populations. Groups of five randomly selected mice were sacrificed at regular intervals over a 60-day period, and test organs were removed aseptically and homogenated separately in sterile saline (5). Suitable saline dilutions were plated on 7H10 agar and incubated at the optimum growth temperature for 2 to 4 weeks. The foot was removed at the ankle, soaked in Zephiran for 15 min (6), washed briefly with sterile saline, and then homogenized in saline with a Virtis high speed blender.

Footpad swelling measurements. The amount of footpad swelling was measured at intervals throughout the growth period using a Schnelltaster dial gauge calipers (5). An increase of 1.8 units (0.18 mm) or more was significant at the 1% level.

RESULTS

Growth of M. kansasii in normal CD-1 mice. Intravenous inoculation of 10^6 viable *M. kansasii* into normal mice resulted in a prolonged systemic infection involving limited growth within both the lung and spleen over the first 20 days, followed by a slow steady decline in viability throughout the remainder of the study (Fig. 1). A few hundred bacilli could be recovered from the footpads of these mice at most times during the study, but there was no significant involvement of the draining popliteal lymph node, nor was there any real increase in foot thickness (<0.2 mm increase).

A persisting infection was also seen when the inoculum was introduced into one hind footpad (Fig. 1). Viable bacilli rapidly appeared in the draining popliteal lymph node, and this population increased considerably before an immune decline was observed, both in the footpad and the draining lymph node. The infection spread

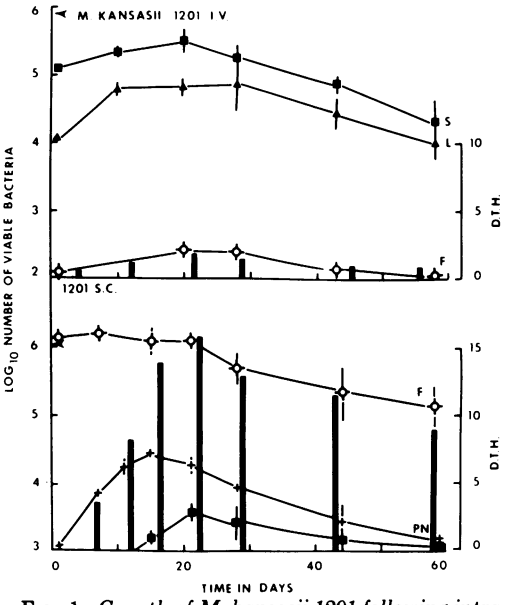

FIG. 1. *Growth of M. kansasii 1201 following intravenous (top) or footpad (bottom) inoculation in normal CD-1 mice. Spleen (S), liver (L), footpad (F), and popliteal lymph node (PLN). The histograms represent the increase in foot thickness of the right hind footpad at increasing time intervals. An increase of 2 or more U is significant ($P < 0.01$). The vertical bars represent standard errors of the mean.*

to the spleen, in which 300 viable bacilli were recovered on day 15, followed by a slow decline. The lung homogenates sporadically contained a few viable *M. kansasii* over the first 15 days of the infection, but the counts were too low and too variable for accurate enumeration. No viable bacilli could be recovered from the contralateral footpad or its draining node.

The injected pad showed a considerable increase in thickness as the infection progressed, and this swelling persisted long after the bacterial population in the footpad began to decline (Fig. 1). None of the mice developed more than a marginal degree of tuberculin hypersensitivity (2.5 μg of PPD-S injected into the left footpad; 1.6 ± 0.4 U at 14 days). Even the injection of 5 μg of the specific PPD-Y resulted in barely significant levels of DTH (1.8 ± 0.6 U on day 20). This low level of hypersensitivity was presumed to be due to the use of the subcutaneous inoculation route.

Growth of M. marinum in normal mice. Groups of normal CD-1 mice were infected in the right hind footpad with about 10^6 viable *M. marinum*, and the growth curves for the three test strains are shown in Fig. 2, together with those for a group of control mice infected by the same route with a similar number of BCG

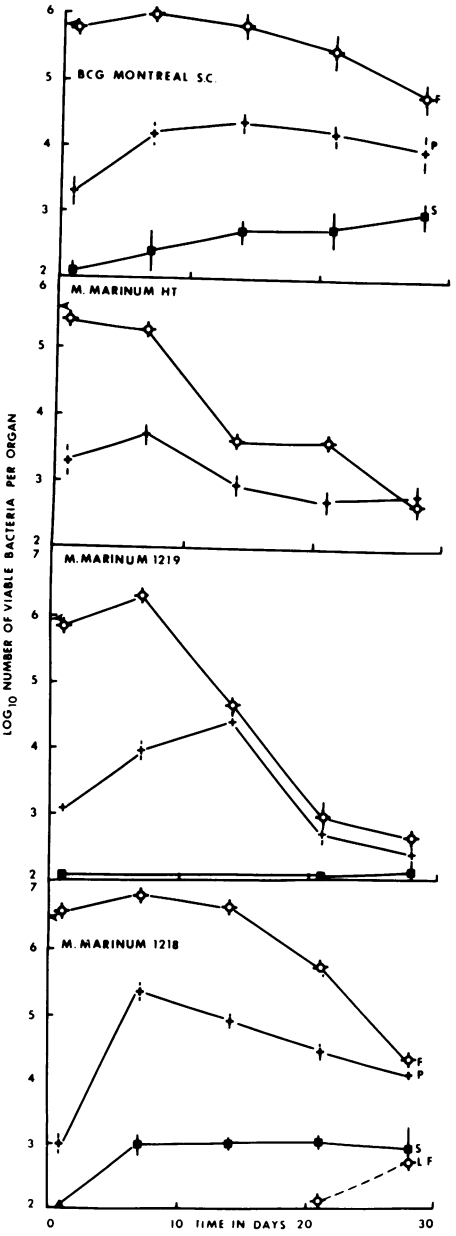

FIG. 2. *Growth of three strains of M. marinum or BCG Montreal following inoculation of about 10^6 viable bacilli (arrow) into the right hind footpads of normal CD-1 mice. Right footpad (F), left (uninoculated) footpad (LF), popliteal lymph node (P), and spleen (S).*

Montreal. Both 1218 and 1219 are so-called "low" temperature strains of mycobacteria (17), but their growth behavior in normal mice clearly differs significantly from each other in several ways. For instance, the peak *M. marinum* 1218 viable counts tended to persist within the footpad for a considerably longer time period than either the 1219 or HT strains. The 1218 infection also quickly passed through the popliteal node to involve other more remote systemic organs (Fig. 2). Viable bacilli were later recovered from the uninoculated, contralateral foot as well as from skin lesions on the tail and ears. Thus, in many regards, the strain 1218 infection tended to resemble that seen earlier in BCG-infected mice (6).

On the other hand, the strain 1219 infection in the footpad was more short lived, with little or no spread by the viable population to the spleen and lungs, although there was considerably early popliteal node involvement. Homogenates prepared from the contralateral foot and from tail skin were negative up to day 50 (Fig. 2). This lack of systemic involvement seemed to correlate with the inability of strain 1219 to survive or multiply at 37 C. Incubation of this organism at 37 C for 5 days was associated with an inability of the culture to grow even slowly at the higher temperature.

There was the possibility that both type strains of *M. marinum* used in the above studies had been maintained on artificial culture media so long that they were no longer mouse virulent. However, a recent human isolate (*M. marinum* strain HT) gave an infection pattern very similar to that for *M. marinum* 1219 (Fig. 2), and this also correlated with the inability of strain HT to multiply or survive at 37 C.

Inoculation of all three *M. marinum* strains into the mouse footpad was associated with a considerable degree of foot swelling over the first 4 days (Table 1). However, the timing and the precise size of the peak footpad reactivity varied considerably and only roughly correlated with the bacterial viable counts obtained for the footpads at that time. For instance, maximum thickness developed 10 to 15 days after the peak viable counts were obtained for *M. marinum* 1219 (Fig. 2). In fact, peak footpad swelling nearly always occurred at a time when the bacterial population within the foot had already passed well into the decline phase (Table 1 and Fig. 3). The maximum size of the footpad reaction varied according to the initial inoculum size. The bacterial populations observed in mouse footpads originally infected with 10^3 or 10^6 viable *M. marinum* 1219 were essentially the same after 10 days, but the maximum footpad thickness observed in the first group of mice was only 3.8 ± 0.6 U on day 15, whereas that for the group originally receiving 10^6 viable bacilli was 12.8 ± 1.0 U (this maximum also developed on day 15; Fig. 3). The number of persisting viable bacilli within the pads of the high and low challenge groups over the 15- to 30-day period

TABLE 1. *Increase over control foot thickness in CD-1 mice inoculated with about 10^6 viable mycobacteria*[a]

Time (days)	*M. kansasii* 1201	*M. marinum* 1218	*M. marinum* 1219	*M. marinum* HT	BCG Montreal
0	0.6 ± 0.2	0.6 ± 0.4	0.8 ± 0.2	0.2 ± 0.4	0.0
1	0.8 ± 0.4	0.8 ± 0.4	1.4 ± 0.2	1.2 ± 0.2	1.2 ± 1.0
2	NT[b]	1.8 ± 0.2	2.8 ± 0.4	NT	NT
4	2.0 ± 1.0	4.8 ± 0.6	4.0 ± 0.8	3.2 ± 0.4	NT
6	4.6 ± 0.7	7.5 ± 1.2	5.6 ± 0.6	6.8 ± 1.2	1.6 ± 0.8
10	8.4 ± 1.4	11.0 ± 1.8	10.2 ± 2.2	NT	NT
14	13.8 ± 1.7	15.8 ± 3.0	11.8 ± 1.8	2.6 ± 0.2	0.8 ± 0.2
21	15.8 ± 2.4	20.8 ± 1.2	9.0 ± 2.4	2.6 ± 0.4	0.4 ± 0.4
28	14.8 ± 2.2	14.6 ± 2.0	6.8 ± 2.2	1.0 ± 0.2	NT
44	17.8 ± 2.0	9.4 ± 1.6	7.2 ± 1.0	1.6 ± 0.2	0.2

[a] Mean of five determinations ± standard error (10 Schnelltaster U = 1 mm). An increase of more than 1.8 U was significant at the 1% level.

[b] NT, Not tested.

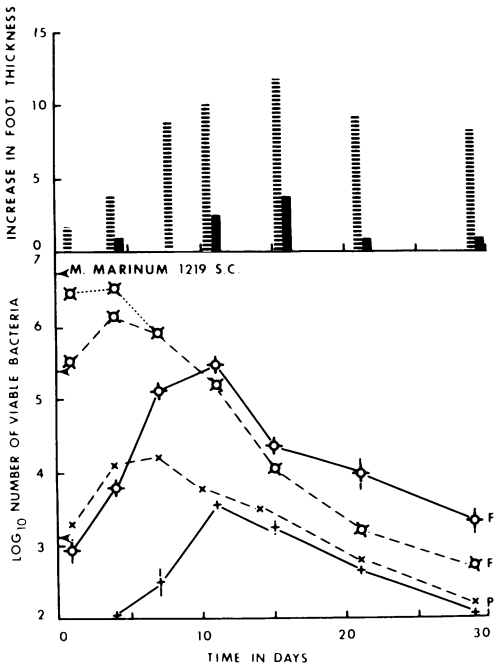

FIG. 3. *Effect of inoculum size on growth of M. marinum 1219 in the footpad (F) and draining popliteal lymph node (P). The dotted line represents the 5 × 10^6 inoculum, the broken lines a 2 × 10^5 inoculum, and the solid lines a 10^3 inoculum into the right hind footpad. The solid histograms represent the increase in thickness of the footpad injected with 10^3 M. marinum on day 0, and the hatched bars represent the corresponding increases in mice injected with 2 × 10^5 viable bacilli.*

was approximately the same, but the amount of footpad swelling in the high dose group continued unchanged for many weeks, whereas that for the 10^3 inoculum declined to nonsignificant levels by day 20. It was not just the continued presence of 10^6 viable mycobacteria within the footpad which brought about this greater degree of swelling, since 10^6 viable BCG Montreal injected into the normal mouse footpad failed to induce any significant increase in thickness at all, whereas that for a similar sized inoculum of *M. kansasii* was substantial (Table 1).

Foot swelling in mice injected with killed M. marinum. Much of the above footpad swelling may have been due to dead mycobacteria or their cell products released into the subcutaneous tissues. There is evidence that the causative agent of this swelling reaction may be relatively heat labile (12), and so the *M. marinum* suspensions were inactivated with 0.5% formalin at 4 C overnight, washed once, and then concentrated to 10^9 to 10^{10} bacilli per ml. Sterility tests carried out on all of the preparations were negative. Groups of normal mice were injected into one hind footpad with 0.04 ml of serial 10-fold saline dilutions of the killed suspensions, and the resulting foot swelling was recorded after 3, 24 and 48 h. The amount of swelling present 24 h after the injection of decreasing amounts of *M. marinum* 1219 suspension is recorded in Fig. 4 and shows that 5 × 10^7 or more dead bacilli induced significant levels of foot swelling, and the response was proportional to the dose used. The time course of this swelling reaction was also interesting. There was an early peak 2 days after injecting 5 × 10^8 dead 1219, with a sharp decline to day 5, followed by a second slower increase again in foot thickness. These pads were still measurably swollen when examined 60 days later. The number of dead bacilli required to induce a significant amount of swelling was relatively large, suggesting that the early response seen in the actively infected mice was not due to dead bacilli also present in the inoculum. There was a considerable difference between the amount of swelling induced by the different dead mycobacterial suspensions. The dead 1219 suspension was far more reactive

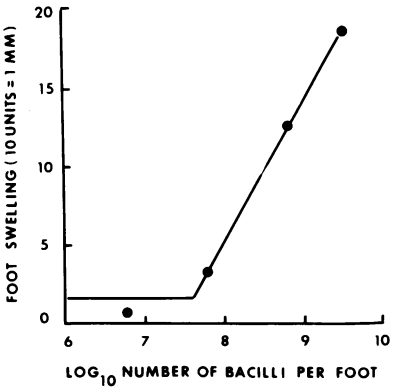

FIG. 4. *Increase in foot thickness 24 h after normal mice were injected in the right hind foot with increasing numbers of dead M. marinum 1219.*

than either 1218 or HT (Fig. 5). This was an interesting reversal of the order seen when living bacilli were injected (Table 1). However, even 10^9 dead BCG Montreal failed to induce any detectable footpad swelling after the first 24 h. Histologically, the foot injected with 10^8 dead *M. marinum* 1219 showed substantial numbers of polymorphonuclear phagocytes together with some mononuclear infiltration and many mature plasma cells appearing both within the footpad and in the draining lymph node 14 days or so later. Large numbers of stainable mycobacteria could still be detected within the footpad even after 28 days, regardless of the strain of organism used.

Growth of an intravenous inoculum of M. marinum in normal mice. Inoculation of 10^5 viable *M. marinum* 1218 via a tail vein in CD-1 mice resulted in the rapid growth of organisms in the spleen over the first 14 days, followed by a slow decline in counts until day 54 (Fig. 6). There was an extensive increase in the number of viable organisms which could be recovered from the hind footpads over the first 30 days, but this was later followed by a substantial decline. At its peak, the footpad population was nearly five times that of the original inoculum. The draining popliteal lymph nodes also became heavily infected early in the experiment, although the viable counts for these organs did not greatly increase further with time. There was a substantial amount of foot swelling observed in these mice after day 10, but unfortunately this parameter of the host response was not quantitated.

Mice infected with *M. marinum* strain 1219 (or HT) developed substantial footpad infections (as well as some tail skin lesions) despite the fact that the systemic bacterial populations declined rapidly with time (Fig. 6). Although the footpad population increased to 10^4 viable bacilli by day 10, the draining popliteal lymph node remained essentially free of viable organisms throughout the experiment. There was also a substantial amount of foot swelling in these mice noted from day 10 onwards.

Growth of M. simiae (habana) in normal mice. Both *M. simiae* (sero-type 1) and *M. habana* gave essentially identical growth curves in CD-1 mice, and since there is some question as to whether these organisms constitute separate species (11), only the *M. habana* data are

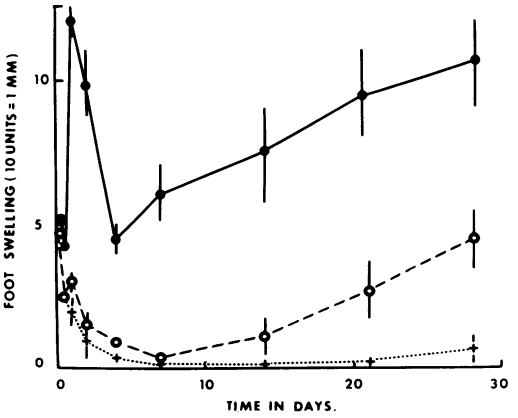

FIG. 5. *Changes in foot thickness of mice injected on day 0 with 6 × 10^8 dead M. marinum 1219 (solid lines) or 5 × 10^7 M. marinum 1219 (hatched line). Mice receiving 5 × 10^7 M. marinum 1218 are represented by the dotted line. Mice receiving 7 × 10^7 BCG showed no detectable swelling after 24 h. The vertical bars represent standard errors of the mean.*

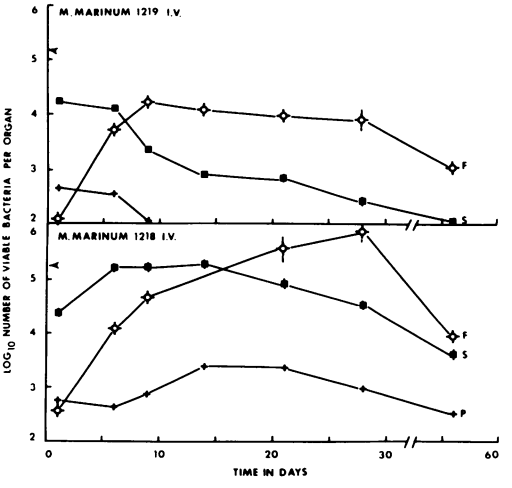

FIG. 6. *Growth of M. marinum 1219 (top) or 1218 (bottom) following intravenous inoculation into CD-1 mice. Footpad (F), popliteal lymph node (P), and spleen (S).*

presented here (Fig. 7). The growth curve for the footpad-challenged animal is remarkable for the continuing persistence of the infection within the footpad itself, as well as in the draining lymph node. Viable organisms could not be recovered from the liver, spleen, or lung at any time during the footpad-induced infection. There was no obvious footpad swelling (<0.15 mm) in these mice.

When the organisms were introduced intravenously into the normal mouse, the organisms accumulated mainly in the liver and spleen, and the infection persisted there with little change for at least 60 days (Fig. 8). The lung population remained relatively low (1 to 300 viable bacilli) throughout the experiment, and no viable organisms could be recovered from the footpads although the popliteal nodes did develop sporadic infections, presumably as a result of the intravenous inoculum. The intravenous mean lethal dose for *M. habana* is in excess of 5×10^8 viable bacilli, and this lack of mouse virulence is consistent with its inability to multiply within the lymphoreticular system. The continuing survival of the inoculum in vivo suggests either that the host's cellular defenses are not stimulated greatly by the infecting bacilli or else this organism is remarkably resistant to the antibacterial action of the host's activated macrophages.

Cross-protection studies in M. marinum-infected mice. *M. marinum* strain 1219, when injected into one footpad, did not spread appreciably to the opposite foot (Fig. 2). Specific challenge of the strain 1219-vaccinated mice could therefore be carried out in the contralateral footpad without the need for a genetically marked strain. Mice were vaccinated with 10^6 viable *M. marinum* 1219 or BCG Montreal in the right hind footpad and then 50 days later

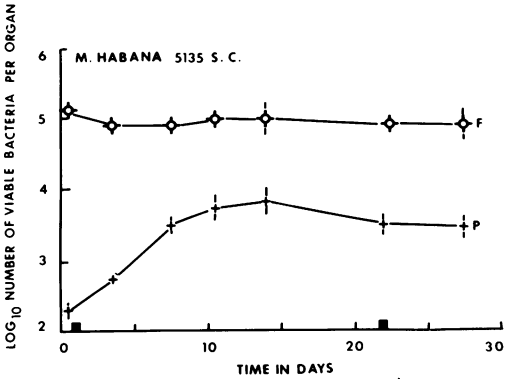

FIG. 7. *Growth of M. habana 5135 following footpad inoculation into normal CD-1 mice. Footpad (F), popliteal lymph node (P). The spleen (■) contained less than 100 viable bacilli throughout the study.*

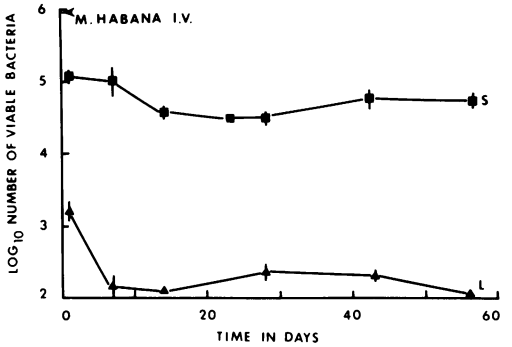

FIG. 8. *Growth of 10^6 viable M. habana 5135 following intravenous inoculation into normal CD-1 mice. Spleen (S), lung (L).*

were challenged in the opposite foot with 10^6 *M. marinum* 1219. A group of normal controls was always included. The growth curves shown in Fig. 9 indicate that little or no increase could be observed in the rate of inactivation of this large superinfecting population by the vaccinated mice compared with that seen in the normal controls. The immunized mice were able to prevent the brief early increase in viable counts which normally occurs within the footpads of the control animals. This relatively disappointing result was presumed to be due to the rapid immune response induced in both vaccinated and control mice by the large challenge infection. When the experiment was repeated using a challenge inoculum of 10^4 viable *M. marinum* 1219, the *M. marinum*-vaccinated mice were seen to develop no more than a twofold increase in viability over the first 8 days, whereas there was a 50-fold increase in both footpad and popliteal lymph node viable populations in the unvaccinated controls (Fig. 10). Such a striking difference in growth was highly significant ($P < 0.01$). Similarly, in the BCG-vaccinated mice, growth of the *M. marinum* within the inoculated footpad was completely inhibited, with an overall 100-fold decrease in viability over the 30-day test period. Interestingly, the strain 1218 vaccine induced a substantially better immune response than did the homologous 1219 vaccine. This greater immunogenicity was ascribed to the systemic 1218 infection which then induced a more effective cell-mediated immunity against the secondary population.

Such a conclusion is also consistent with the growth data obtained using an *M. tuberculosis* Erdman challenge. Both the *M. marinum* vaccines induced an immune response capable of inhibiting the growth of this highly virulent challenge organism in vivo (Fig. 11). However, the 1218-vaccinated mice also prevented the spread of the virulent infection from the footpad

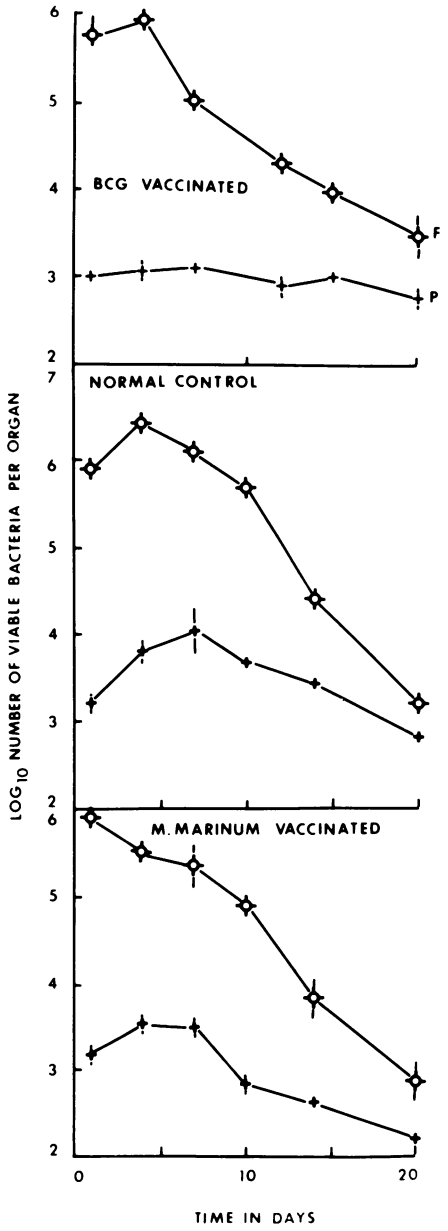

FIG. 9. *Growth curves for 10^6 M. marinum 1219 inoculated into the left hind footpads of mice vaccinated 50 days previously with 10^6 viable M. marinum 1219 (bottom) or 10^6 viable BCG (top). Normal unvaccinated controls challenged in the left footpad are shown in the middle section.*

to the spleen and the lung, whereas there was a transitory increase in viable Erdman present in the spleens of the 1219 vaccinated animals.

DISCUSSION

The photochromogenic mycobacteria include a number of potential human pathogens which are capable of multiplying within the lymphoreticular organs of normal mice. They cross-protect the mice against a subsequent challenge with either human and bovine strains of tubercle bacillus (3, 9). Evidence has been presented by several groups that *M. marinum* and *M. bovis* (BCG) cross-react in this way in vivo and claims have also been made that some cross-protection is even generated against an *M. leprae* challenge (13). This finding has not been mirrored clinically by a significant protective potential in BCG-vaccinated individuals against human leprosy (1, 15). Such discrepancies may be due to technical factors involved in the demonstration of acquired anti-mycobacte-

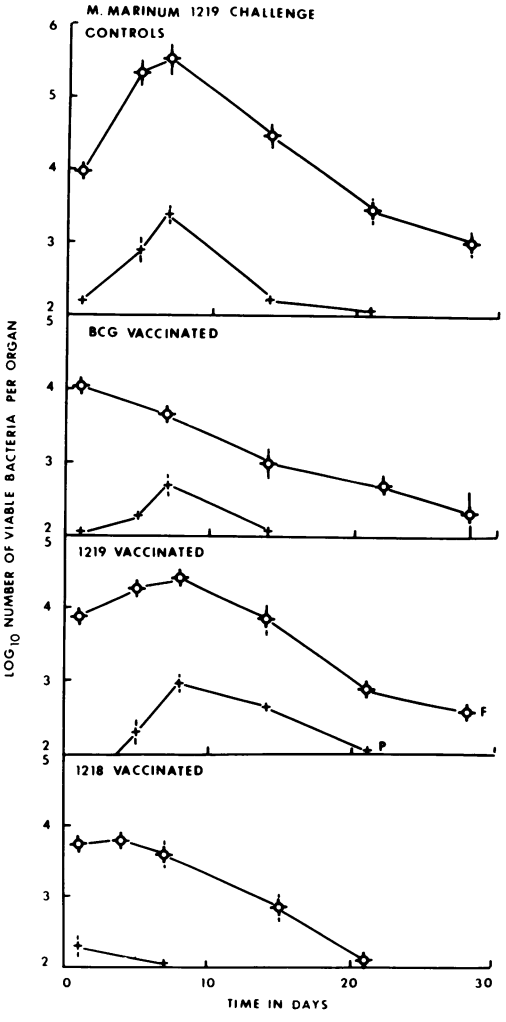

FIG. 10. *Growth of 10^4 viable M. marinum 1219 in the left hind footpads of mice vaccinated 50 days previously with 10^6 M. marinum 1218 (bottom), 1219 (second from the bottom), BCG Montreal (second from the top) or with saline (top).*

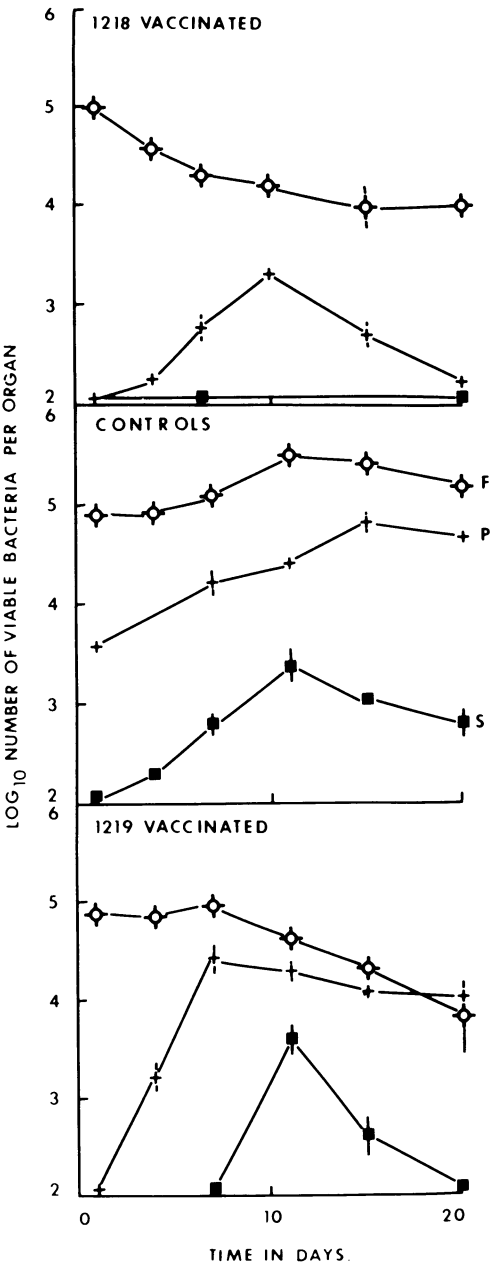

FIG. 11. *Growth of 10^5 viable M. tuberculosis Erdman in the left hind footpads of mice vaccinated 50 days earlier with 10^6 M. marinum 1219 (bottom) or 1218 (top) or saline (middle).*

rial resistance in the laboratory model. Many strains of tubercle bacilli are extraordinarily resistant to inactivation, and it may be quite difficult to demonstrate an antituberculous response unless differential microbial enumeration methods are available (4). Such counting methods tend to be both tedious and time consuming, and so the suggestion of Ng et al. (13), that the degree of footpad swelling in *M. marinum*-infected mice could be used to monitor the immune response in suitably vaccinated mice, is at first sight an interesting one. These authors reported that immunity could be deduced simply from the absence of foot swelling in the challenged host. However, data presented here suggests that considerable care should be exercised in interpreting such protection data. Extensive edema and cellular infiltration can occur within the footpad even following the injection of dead bacilli, and this swelling may have little relation to the number of viable bacilli present within the footpad at any one time. Such swelling may arise from the release of inflammatory products from either metabolically active or dead cells. Thus reliance on the degree of foot thickening as a measure of the amount of bacterial growth occurring in the footpad could give rise to misleading conclusions regarding the host-parasite interactions involved unless enumeration of the bacterial population within the infected footpads is also carried out in both the vaccinated and control animals.

Shepard and Haboes (14, 16) and Ng et al. (13) have suggested that *M. marinum* could provide a useful experimental tool for studying the role of normal host defenses in limiting the growth of *M. leprae* within the mouse footpad. However, the present data suggests that *M. marinum* is not particularly suitable for this type of study. Some strains (37 C adapted) give rise to considerable systemic involvement which in no way resembles the type of infection seen with *M. leprae*. In addition, even *M. marinum* strain 1219 induces an acute inflammatory response within the footpad quite unlike that seen in *M. leprae*-infected footpads. This reaction seems to be a predominantly polymorph response, and the chronic ulcerative lesions are quite atypical of the *M. leprae* response. *M. marinum* also induces a clearcut immune response 10 to 14 days after challenge, with viable bacilli largely disappearing from the footpad within weeks of infection. These anomolies are all substantial, suggesting that *M. marinum* is not a suitable model for studying *M. leprae* infections in the mouse. Perhaps strains which behave more like *M. simiae* or *M. habana* in the mouse footpad may prove to be more suitable for such studies, and work along these lines is currently in progress.

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LITERATURE CITED

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