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# Immunity to *Mycobacterium leprae* Infections in Mice Stimulated by *M. leprae*, BCG, and Graft-Versus-Host Reactions

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Infections of mice with *Mycobacterium leprae* in one rear foot pad immunized them against a second infection in the other rear foot pad. Purified bacilli harvested from the first infection also produced immunity when injected into the foot pads of previously uninfected mice. Injections of BCG afforded similar protection, but had no adjuvant effect on *M. leprae*. *M. duvali*, a cultivable mycobacterium that is reported to be more closely related antigenically to *M. leprae* than BCG is, provided much less protection against *M. leprae* challenge than BCG did. Moreover, when *M. duvali* was mixed with BCG, it was not any more effective than BCG alone. Graft-versus-host reactions, induced by injections of parental spleen cells into F1 hybrids, provided no protection against *M. tuberculosis* and *M. marinum* challenge. They gave moderate protection against *M. leprae* in one experiment but not in another with a different schedule. Allogenic spleen cells had a protective effect when injected locally into the infected foot pad. The effect produced by these injections of spleen cells was a delay in the appearance of bacterial growth; however, there was no decrease in the rate of logarithmic growth when it did appear and no reduction in the eventual plateau level.

In earlier studies we had found that immunity against *Mycobacterium leprae* infections in mice could be induced regularly with intradermal injections of *M. bovis* (strain BCG) (16) and cell walls of BCG when attached to oil droplets (26). In a single reinfection experiment, in which the first antigenic exposure occurred as usual via the foot pad route, immunity was not observed (16). More recently, however, others have reported immunity in mice on reinfection with *M. leprae* (9, 11). Resistance against *M. leprae* infections has also been found to result from infections with *Toxoplasma gondii* and *Besnoitia jellisoni* (10); these two parasites presumably have no antigenic relationship to *M. leprae*, but they cause persisting infections and produce nonspecific resistance against salmonella infections and certain tumors, probably by activating macrophages (10).

To investigate some of the apparent discrepancies among these findings, we have undertaken new studies. Reinfection immunity has been explored more carefully and compared with that induced by injections of live BCG. The immunity induced by injections of *M. duvali* has been explored; this mycobacterium has been reported to be antigenically more closely related than BCG to *M. leprae*, as judged by lymphocyte transformation tests (6). In addition, graft-versus-host (GVH) reactions have been studied as a possible source of nonspecific immunity against *M. leprae*.

## MATERIALS AND METHODS

The methods for the infection of mice have been described (15). In brief, 5,000 *M. leprae* from a strain in mouse passage were inoculated into a rear foot pad. The course of the infection was then followed by counting the acid-fast bacilli (AFB) in suspensions of harvested foot pad tissue (24). Unless otherwise stated, each point on the growth curves represents the number of AFB per mouse in a pool of the tissues of four mice. The lower limit of sensitivity in harvests of individual mice (the value corresponding to one AFB in the approximately 66 fields examined) is about $10^{4.8}$ AFB/mouse. Similarly, the lower limit for a pool of eight mice is about $10^{3.9}$ AFB/mouse, since the foot pad tissues of the eight mice are suspended in nearly the same volume as those from one mouse. Intradermal vaccinations were given into the flank of mice about 7 weeks of age after the fur was removed with small-animal clippers. Enough vaccine was given through a 30-gauge needle to raise a small but distinct bleb; the volume averaged about 0.01 ml. For such injections, the unanesthetized mouse is held in one hand by the skin at the back of the neck and by the tail. The flank skin is

then stretched by placing the upper hind leg under the fourth finger. In the unanesthetized mouse the abdominal musculature produces adequate counterresistance to the needle. With practice, satisfactory injections can be made consistently, but when a bleb is occasionally not raised because the needle is too deep, the injection is repeated. CFW and A strain mice were obtained from the animal facilities at the Center for Disease Control; the other inbred and the hybrid strains were purchased from the Jackson Laboratories, Bar Harbor, Maine. Female mice were used throughout.

GVH reactions were induced by the injection of parental spleen cells into F1 hybrids. The technique of Gleichman et al. was used to produce chronic but nonfatal GVH reactions (5); in this technique the injections are given four times at weekly intervals. The combinations used here had been studied by those authors; C3H/He and DBA/2 are among the strains giving good growth of *M. leprae* (22). Donor mice were killed by cervical dislocation, the spleens were placed in Hanks balanced salt solution (BSS) in a beaker, the cells were expressed with the ends of two glass slides, and the clumps were broken up by aspiration through a 25-gauge needle. The cells were washed twice by centrifugation and resuspended in BSS. For systemic GVH reactions, $5 \times 10^7$ spleen cells were given intraperitoneally. In other mice, local injections of $1 \times 10^7$ spleen cells were given into the foot pad on the same schedule; it was hoped that, by the injection of lymphocytes into the foot pad, a local GVH reaction could be set up that would more effectively activate local macrophages. After all injections into normal foot pads, a local infiltrate of leukocytes develops and persists for about 1 week. For the first 2 days or so polymorphonuclear cells predominate, but lymphocytes and macrophages then become the dominant cells. The cells in the infiltrate would be readily accessible to injected cells that remained in the foot pad.

The BCG, *M. duvali*, and *M. tuberculosis* cultures were maintained on Lowenstein-Jensen medium. For use in the experiments, cultures were grown in Tween-albumin medium (Tb-Tween; Difco), washed twice in phosphate-buffered saline with 0.05% Tween 80 (PBS-Tw), and preserved at −60°C. To obtain suspensions of high viability, the BCG cultures were harvested at 13 days, which was well before maximal turbidity. The *M. duvali* cultures were harvested at 7 days, but this mycobacterium does not grow well in the Tween-albumin medium. Attempts to find another medium that gave dispersed growth were not successful. The amount of bacterial growth of BCG and *M. duvali* was standardized on the basis of the volume of bacterial sediment in Hopkins tubes after centrifugation at 2,000 × *g* for 60 min. Repeated measurements with BCG cultures have shown that $1 \times 10^9$ BCG have a sedimented volume of approximately 10 μl. To facilitate comparison between *M. leprae* and BCG, the concentration of the vaccine is given as the number of AFB injected.

The *M. leprae* in harvested foot pad tissues was purified by a method previously described (21) in which 0.125% trypsin is used for 5 min at 37°C and self-aggregation is used to eliminate collagen fibers. With this process there is very good recovery, the *M. leprae* have a high degree of purity, and there is no decrease in viable bacilli measurable by mouse inoculation. In the *M. duvali* experiment, the local induration was measured periodically, as were the regional (inguinal) lymph nodes. This lymph node can be visualized in a closely clipped mouse, when the skin is wet with alcohol, as a slightly bluish area in the surrounding yellow fatty tissue; the node is made more obvious when it is pushed gently. Two diameters at right angles were measured with a millimeter rule, and the results are presented as the average of the two diameters.

## RESULTS

**Reinfection and vaccination.** In our earlier experiments with *M. leprae*, the lack of homologous immunity observed on reinfection had stood in contrast with the consistent immunity seen with injections of live BCG (16); among the explanations for this seeming paradox were that the *M. leprae* infectious process somehow masks the systemic immune response against *M. leprae* although it is able to shut off multiplication of *M. leprae* locally, that the foot pad route is not as effective for immunization as is the intradermal route, and that *M. leprae* lacks the well-known adjuvancy of BCG.

Accordingly, in the first experiment to be described, the protective effect of a primary foot pad infection was compared with that induced by the injection of the same number of *M. leprae* into the foot pad; immunizing injections into the foot pad were used to compare *M. leprae*, BCG, and mixtures of *M. leprae* and BCG, and BCG injections were used to compare the foot pad route with the intradermal route. The design of the experiment is presented in Table 1 and Fig. 1 and 2. At the beginning of the experiment, 600 CFW mice of the same age were caged and their cages were randomized. Numbers 1 to 260 and three smaller groups were inoculated with $5 \times 10^3$ *M. leprae* in the right hind foot on day 0. The timing of the remaining injections was determined by the *M. leprae* growth curve in the primary infection (Fig. 1). On day 225, 175 mice from numbers 1 to 260 were killed, their foot pad tissues were harvested, and the contained *M. leprae* were purified by the trypsin method and adjusted to a concentration so that the same number of *M. leprae* harvested per foot pad ($2.4 \times 10^6$) was contained in the 0.03 ml injected. This amount was given on the same day into the right hind foot of the previously uninoculated mice indicated. At the same time, $1 \times 10^7$ BCG were given by the routes indicated. The mixture of *M. leprae* and BCG was made before injection.

TABLE 1. *Results of the experiment involving reinfection and vaccination*

| Mouse no. | Injections[a] on day: | | | | Harvests ($\times 10^5$) on day: | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 (RHF[b]) | 225 | | 254 (LHF[b]) | 450 (LHF) | 540 (LHF) | 450 (RHF) | 540 (RHF) |
| | | RHF[c] | ID[c] | | | | | |
| 1-260 | ML | | | | <0.09 | <0.08 | 11.3[d] | 6.52[d] |
| 261-310 | | | | ML | 12.5[d] | 12.9[d] | <0.07 | <0.09 |
| 311-360 | ML | | | ML | 0.42[d] | 1.28[d] | 28.5[d] | 10.7[d] |
| 361-390 | ML | BCG | | ML | 0.41 | <0.05 | 16.5 | 4.58 |
| 391-420 | | ML + BCG | | ML | 1.77 | 2.28 | 16.8 | 2.53 |
| 421-450 | | ML | | ML | 0.30 | 0.42 | 5.26 | 4.57 |
| 451-480 | | ML | | | <0.05 | <0.04 | 9.19 | 0.36 |
| 481-510 | | BCG | | ML | 5.58 | 0.48 | 3.30 | 1.06 |
| 511-540 | | BCG | | | ND | ND | 9.52 | 2.79 |
| 541-570 | | | BCG | ML | 0.13 | 5.65 | ND | ND |
| 571-600 | ML | | BCG | ML | <0.05 | 0.17 | 1.47 | 5.93 |

[a] RHF, Right hind foot; LHF, left hind foot; ID, intradermal; ND, not determined.
[b] ML = $5 \times 10^3$ *M. leprae*.
[c] BCG = $1 \times 10^7$ BCG; ML = $2 \times 10^6$ *M. leprae*.
[d] Averages of individual harvests. All other values are for pools.

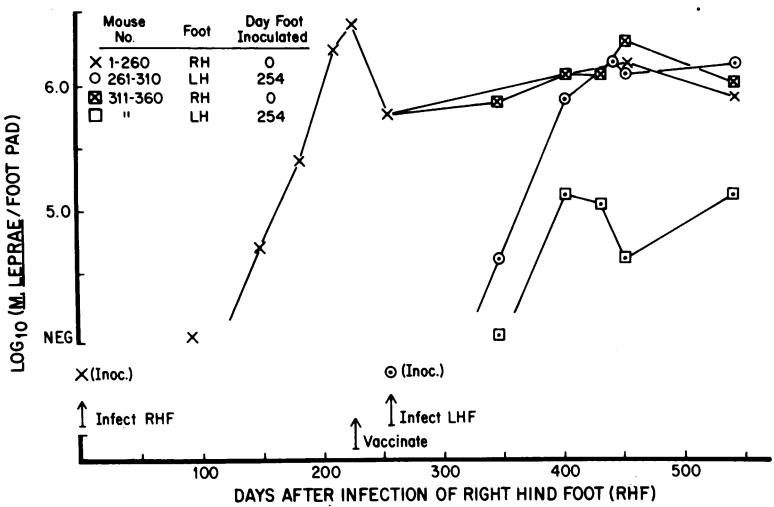


FIG. 1. *M. leprae growth curves of reinfection and vaccination experiment. The points represent the average harvest per mouse in a pool of four mice, except as follows: 225 days, 175 mice; 254 days, 9 mice; and 450 and 540 days, average of counts for eight individual mice.*

On day 254, nine more mice from numbers 1 to 260 were harvested to provide the inoculum for the left hind feet of the mice indicated. These consisted of numbers 261 to 310 (left hind foot infection controls; Fig. 1), numbers 311 to 360 (reinfected groups), and most of the groups in numbers 361 to 600 (the inoculated groups are indicated in Table 1). The *M. leprae* growth curve in the left hind feet was then followed in numbers 261 to 310, and at 450 days when the plateau was reached eight mice in each group were harvested. The harvests were repeated at 540 days.

The growth curves in Fig. 1 are typical, except that the drop in numbers immediately after the shutoff of logarithmic growth in the right hind feet was somewhat greater than usual.

The right hind foot harvests (Table 1) show that *M. leprae* persisted until day 540, and it did so whether it had grown to plateau levels or whether it had been injected at plateau levels. BCG also persisted in the inoculated foot. Thus the antigenic stimulus was provided until the end of the experiment.

The left hind foot harvests show that infection immunity was clear-cut. There was little growth delay in the reinfection, but the plateau

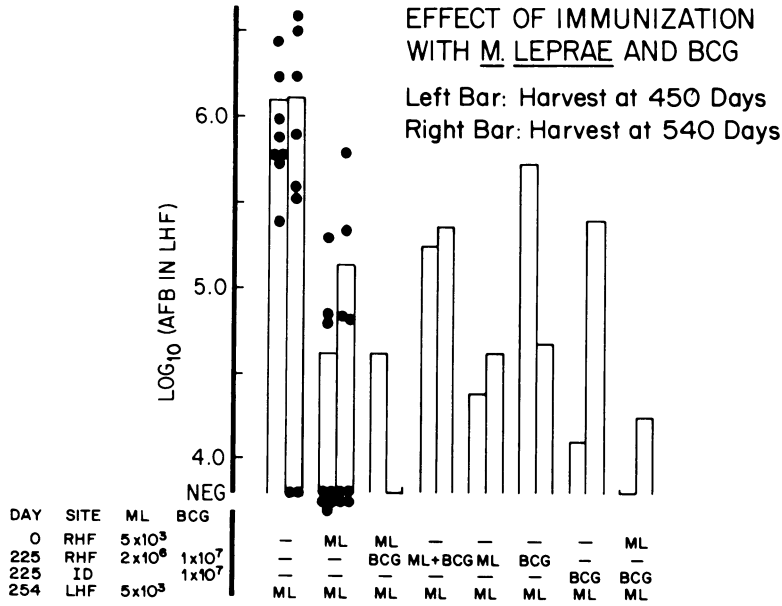


FIG. 2. *M. leprae harvests in reinfection and vaccination experiment.*

was imposed at a lower level, that is, when the *M. leprae* growth curve reached $10^{5.0}$, or about $^1/_{10}$ that in the controls. The significance of the differences in individual harvests between these two groups (Fig. 2) was determined by the two-sample rank test; at 450 days the *P* value was less than 0.01, and at 540 days the *P* value was between 0.05 and 0.10. The results in the other groups suggest the following. (i) Injection of *M. leprae* into the foot pad led to no more immunity than the presence of the same number as a result of the infectious process. (ii) The mixture of BCG and *M. leprae* was no more effective than either given alone into the foot pad. (iii) In *M. leprae*-infected mice, BCG injected into the infected foot possibly increased the immunity, but it was not noticeably more effective than BCG given intradermally into the opposite flank; both groups had one completely negative group harvest. In the latter group, adjuvant activity was unlikely since the two antigens drained into different regional lymph nodes. As indicated below, however, comparisons between values for pools of mice showing partial protection are somewhat hazardous.

***M. duvali* as vaccine.** In the next experiment, *M. duvali* was tested and compared with BCG. The undiluted vaccines (1:1) contained 0.1 $\mu$l of bacterial mass ($1 \times 10^7$ organisms in the case of BCG) in the 0.01 ml administered. Dilutions of 1:2 and 1:4 were also administered singly and in combinations. There was one unvaccinated control group for each vaccine group, and one control group received diluent only (PBS-Tw). All of the vaccines were given intradermally 28 days before the challenge with *M. leprae*. This is the design we had used in earlier vaccine experiments (16).

The size of the local induration and the regional lymph node was measured in 10 mice from all vaccinated groups, from one unvaccinated group, and from the group receiving diluent only. The measurements were begun 28 days after vaccination, and they were repeated in the same mice, if surviving, at 28-day intervals. In Fig. 3, the results are shown for only four groups. With the *M. duvali* vaccine given alone, many of the mice did not have local reactions, but with the BCG vaccine local reactions averaged 1 to 2 mm at all times studied. There was a suggestion that the mixture gave less local reactions at the first interval studied. No local induration was present in the two control groups. The size of the draining inguinal node, normally about 2 mm, increased to 6 to 8 mm in the mice receiving BCG alone; the increase in those receiving *M. duvali* vaccine was less marked, and the sizes reverted toward normal at the later readings. The mixture appeared to give less enlargement than did BCG alone. The 1:4 dilution of BCG vaccine gave distinct enlargements in most mice, and the average rose to 5.3 mm at 12 weeks; the 1:4 *M. duvali* vaccine gave no distinct enlargement, and the mixture produced enlargements that

averaged somewhat less than that with BCG alone.

The growth curve of *M. leprae* was again followed by harvests from pools of control mice and, when the curve passed $10^{6.0}$, eight mice were killed from each group for counts of AFB; the counts were repeated 90 days later (Fig. 4 and Table 1). The counts were carried out on individual mice from four groups, and the rest were counted as pools. The undiluted *M. duvali* vaccine gave moderate but significant protection, which, in turn, was significantly less than that provided by the BCG (Table 2). The mixture of the two vaccines, each in a final dilution of 1:2, was not any more effective than undiluted BCG. The *M. duvali* vaccine was not effective when diluted 1:2 and 1:4. Dilution of the BCG did not appear to reduce its potency.

The distribution of values in the individual counts revealed that the counts on pooled tissues could give a misleading picture. Thus, in groups B and D, one or two mice had $10^{5.3}$ or more AFB/mouse, whereas the remaining mice had no detectable bacilli.

**GVH reactions.** C3H/J × DBA2/J F1 hybrids (C3D2) received intraperitoneal injections of 5 × $10^7$ parental cells (C3 or D2), allogenic cells (A/He), syngenic cells (C3D2), or BSS according to the schedule shown in Fig. 5. In the first part of the experiment they were challenged

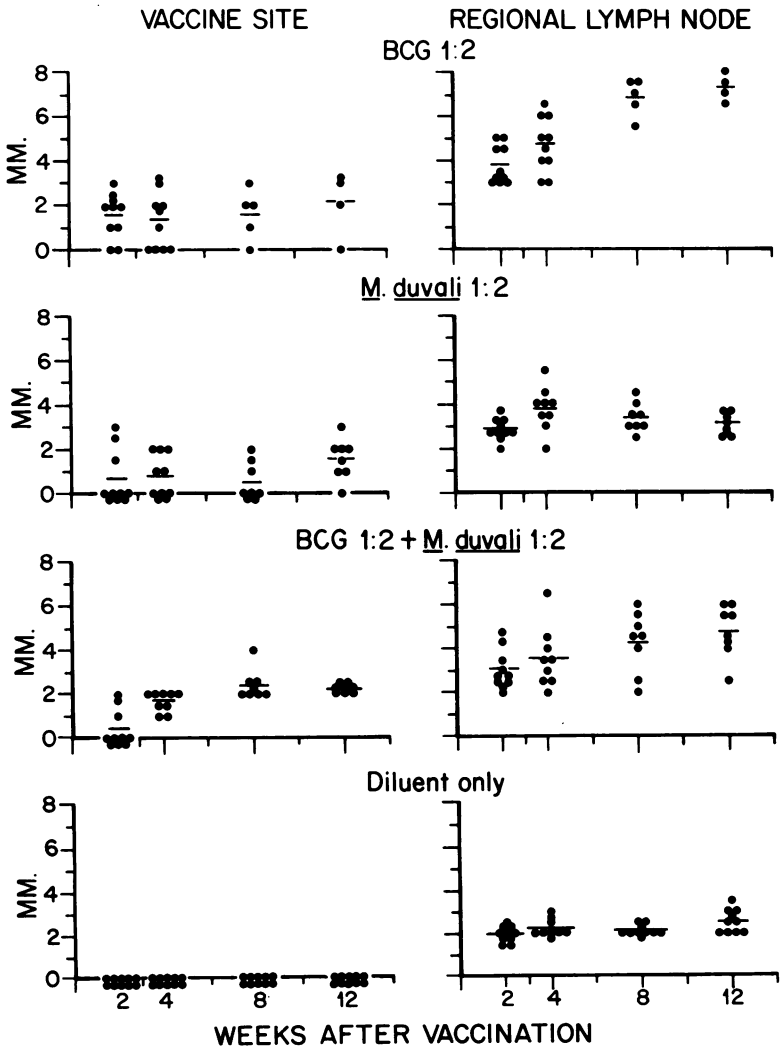


FIG. 3. *Induration at vaccine site and size of regional lymph nodes after intradermal vaccination with BCG, M. duvali, and mixtures of the two.*

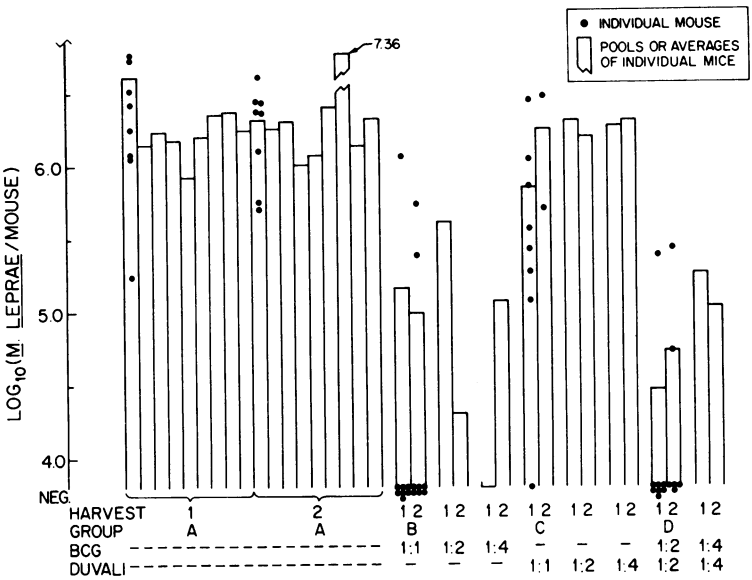


FIG. 4. *M. leprae harvests in M. duvali vaccine experiment.*

TABLE 2. *P values for the differences between groups in M. duvali experiment calculated by two-sample rank test*

| Group | Probability (*P*) values for the differences | | | |
|---|---|---|---|---|
| | Nil (A) | BCG (1:1) (B) | *M. duvali* (1:1) (C) | BCG (1:2) + *M. duvali* (1:2) (D) |
| First harvest | | | | |
| A | | | | |
| B | <0.001 | | | |
| C | 0.01 | 0.012 | | |
| D | <0.001 | 0.500 | 0.002 | |
| Second harvest | | | | |
| A | | | | |
| B | <0.001 | | | |
| C | 0.500 | <0.044 | | |
| D | <0.001 | 0.475 | 0.036 | |

intravenously on day 0 with 0.03 mg of virulent tubercle bacilli (strain H37Rv). The groups contained 17 to 20 mice. There was no important difference among the groups.

In another part of the experiment, mice were challenged by the injection of *M. marinum* (strain Balnei X) into the right hind foot pad. The progress of the experiment was monitored by measurements of foot pad swelling. Some of the mice had received the same materials intraperitoneally, and others had received $1 \times 10^7$ cells in a volume of 0.03 ml into the right hind foot pad on the same schedule. No important differences among the groups were observed.

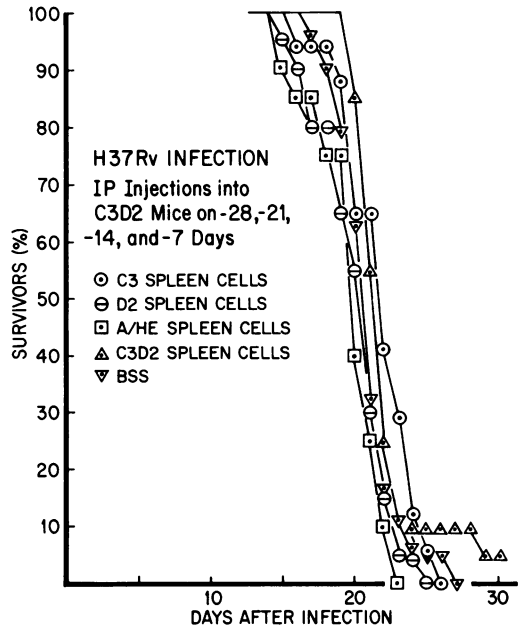


FIG. 5. *Effect of GVH reactions on survival of mice after intravenous challenge with M. tuberculosis, strain H37Rv.*

In another experiment (Fig. 6 and 7), *M. leprae* was used as the challenge. The spleen cells were injected on −10, −3, +4, and +11 days. After intraperitoneal injections (Fig. 6) the growth of *M. leprae* was delayed in the group that received parental cells (C3), but not in the various controls. After local foot pad in-

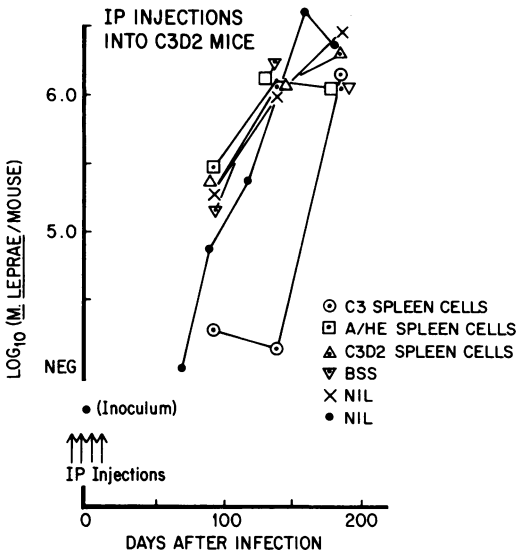


FIG. 6. *Effect of GVH reactions on multiplication of M. leprae.*

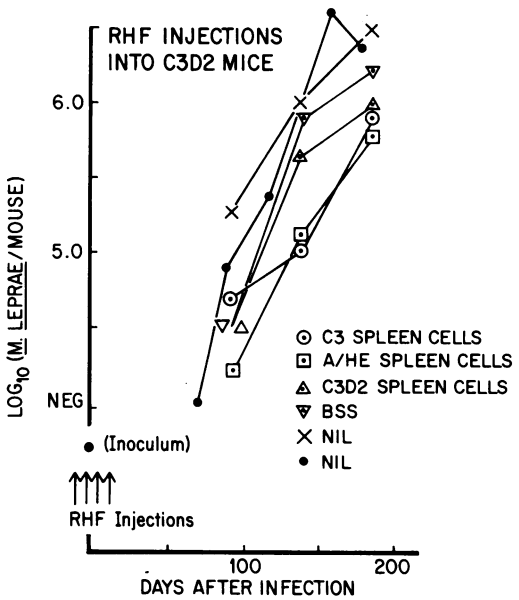


FIG. 7. *Effect of local injections of parental and allogenic spleen cells on multiplication of M. leprae in F1 hybrid mice.*

jections of cells, the growth of *M. leprae* was delayed in the group receiving parental cells and in the group receiving allogenic (A) cells, but not in the other groups (Fig. 7). The amount of growth delay is tabulated in Table 3. The experiment is similar in form to the kinetic method used for studying antileprosy drugs (18); extensive experience with this method has led to the conclusion that growth delays of 60 days or more are significant. After the initial delay, the growth of *M. leprae* took place at normal rates and normal plateau levels were reached. Such a result would have been produced by an effect exerted only in the very early stages, just after inoculation.

In a repeat experiment the cells were given either just before inoculation of *M. leprae* (−24, −17, −10, and −3 days) or later at the beginning of the logarithmic phase of growth (+63, +70, +77, and +84 days). With the latter schedule, a protective effect that was capable of acting against *M. leprae* already multiplying in the foot pad could be detected. The results of this experiment also are shown in Table 3. When given before infection, none of the intraperitoneal injections was effective, but the local injection of C3 cells caused 54 days of growth delay. When given after infection, again none of the intraperitoneal injections was effective, but the allogenic cells given locally caused 69 days of growth delay. In AKD2 hybrids, intraperitoneal injection of D2 cells was not effective.

TABLE 3. *M. leprae growth delay in F1 hybrid mice receiving parental or control cells*

| Expt | Recipient | Day of injection[a] | Cells injected | Days of growth delay[b] with injection given | |
|---|---|---|---|---|---|
| | | | | i.p. | RHF |
| 1 | C3D2 | −10, −3, +4, +11 | C3 | 65 | 59 |
| | | | A | −13 | 59 |
| | | | C3D2 | −3 | 27 |
| | | | None[c] | 2 | 21 |
| 2 | C3D2 | −24, −17, −10, −3 | C3 | 15 | 54 |
| | | | D2 | 18 | 27 |
| | | | A | 13 | 33 |
| | | | C3D2 | 39 | 12 |
| | | | None | 43 | 13 |
| 2 | C3D2 | +63, +70, +77, +84 | C3 | 40 | 34 |
| | | | D2 | 10 | 24 |
| | | | A | 0 | 69 |
| | | | C3D2 | 20 | 27 |
| | | | None | 0 | 27 |
| 2 | AKD2 | −24, −17, −10, −3 | D2 | 9 | |
| | | | AKD2 | −7 | |
| | | | None | 11 | |

[a] Relative to the day of inoculation of *M. leprae* into the right hind foot.

[b] Relative to the control curve (*M. leprae* only). The level used in experiment 1 was $10^{5.5}$ (104 days in control). The level used in experiment 2 was $10^{5.5}$ (128 days in C3D2 control and 145 days in AKD2 control). i.p., Intraperitoneal; RHF, right hind foot.

[c] Diluent (BSS) only.

## DISCUSSION

In a foot pad infection with a fast strain (26) of *M. leprae* in previously uninfected mice, logarithmic multiplication continues until the number of *M. leprae* passes a level of about $10^{6.0}$. Logarithmic multiplication then stops abruptly, and the number of AFB falls a little. (The number of viable *M. leprae* decreases markedly at this time, as measured by bacterial morphology [23] and by mouse inoculation [20].) The total number of AFB then remains more or less constant for a year or so. The abrupt termination of logarithmic multiplication of course suggests an immune response. The histological picture, as studied by electron microscopy (12), is compatible with this interpretation. During the logarithmic phase, the *M. leprae* are located free in the cytoplasm of macrophages that are "quiet" in the sense that they contain few lysosomes, vacuoles, or residual bodies. At the termination of logarithmic multiplication, there is lymphocytic infiltration, and the degenerating *M. leprae* appear degenerated and they are found surrounded by membranes in activated macrophages that contain many lysosomes and vacuoles. Supposedly the mitotic steps resulting in the formation of sensitized T-lymphocytes take place in the regional (popliteal) lymph node, and it had seemed incongruous that the sensitized lymphocytes could not find their way to the second infection also.

The present results, as well as those of Kawaguchi (9) and Levy (11), make clear that reinfection immunity to *M. leprae* can manifest itself. Our previous failure to demonstrate reinfection immunity (16) is perhaps explainable by the timing of the challenge infection. In the earlier work we had not monitored the primary *M. leprae* growth curve closely, and the challenge inoculum was given at 8 months, a time that may have been several months after the attainment of plateau levels in the primary infection. Kawaguchi (9) and Levy (11) have shown that if the challenge infection is given too early, reinfection immunity is not manifested. The duration of reinfection immunity after plateau in the primary infection has not been determined, however. The reappearance of solid-staining bacilli suggests that the immunity does not last long (23). Inconsistencies between experiments also occur. Thus, in the present experiment, the injection of $2 \times 10^6$ *M. leprae* into the foot pad 28 days before challenge appeared to be effective, and as effective as the same number of organisms present as the primary infection. In another experiment, $3 \times 10^6$ *M. leprae* from a human biopsy specimen injected into a foot pad 28 days before challenge did not afford significant immunity, although the same number of organisms injected intradermally did (19). In that experiment, although the bacilli were purified by the same method, the proportion of viable bacilli was probably lower (about 7 days elapsed from the removal of the specimen to the inoculation of the mice); moreover, there could have been antigenic differences between the immunizing and challenge strains.

The results of the first experiment suggest that, for immunization with live BCG, the intradermal route is more effective than the foot pad route. Later work has confirmed this and has shown the same superiority for the intradermal route with live *M. leprae* and with a mixture of live *M. leprae* and BCG (19). Many explanations appear possible at this stage. In our experience an average of about 90% of the *M. leprae* injected into the foot pad leave the foot pad immediately, presumably by escaping up the lymphatic channels to the popliteal lymph nodes. When washed suspensions of the carbon particles of India ink are injected into the foot pad, much of the particulate matter reaches the popliteal lymph node within a few minutes, although much remains in the foot pad dermis or in the connective tissue around the vessels and nerves and between the muscle bundles. (The substantial drainage from the foot pad into the popliteal lymph node has been made use of in a technique for producing GVH reactions in this node [3].) In contrast, after intradermal injections of carbon particles into the flank, all of the particles remain at the site of injection and no drainage to the regional (inguinal) node can be demonstrated grossly or histologically. After intradermal injections of BCG, eventual drainage of antigen to the inguinal nodes is indicated by the lymph node enlargements that occur over the course of weeks and months (Fig. 3). Other differences between the flank and the foot pad include the temperature (about 35°C in the flank skin versus 30°C in the foot pad [17]) and the content of mast cells (many more in the foot pad [4]).

The results of the first experiment also emphasize the difficulty encountered in attempts to demonstrate an adjuvant effect by BCG in vaccines against *M. leprae*. The difficulty arises because the adjuvant (BCG) is active by itself. The immunity associated with a foot pad infection with *M. leprae* appeared to be augmented as much by intradermal injections into the opposite flank as by injections into the infected foot pad. Adjuvancy would not be expected when the adjuvant and the antigen drain into different lymph nodes, so the appar-

ent increased protection afforded when BCG was given into the opposite flank presumably came about because the BCG and the *M. leprae* were independently effective, for example, by sensitizing independent populations of T lymphocytes. An early experiment purporting to show an adjuvant effect of BCG on heat-killed *M. lepraemurium* as vaccine against *M. lepraemurium* (8) did not take this possibility into account. Another complicating factor in the interpretation is that *M. leprae* itself has adjuvant activity, although somewhat less than that of *M. tuberculosis* (27). Adjuvant activity is common among the mycobacteria, and it is not yet evident whether admixture with BCG can be used to increase the response against another intact mycobacterium. BCG injections have been tried as a therapeutic measure in lepromatous leprosy (14), and the results were suggestively beneficial. To learn whether a beneficial therapeutic effect of BCG in patients is caused by its adjuvant activity may be difficult, however, because *M. leprae* is widely distributed in lepromatous disease and most skin accessible to vaccine injections would probably contain some *M. leprae*.

In the experiment with *M. duvali* some problems were encountered that may complicate the screening of other cultivable mycobacteria as candidates for leprosy vaccine. The *M. duvali* vaccine stimulated little tissue reaction at the vaccination site and minimal enlargement of the regional lymph node, and it provided little protection against *M. leprae* infection. It is possible that growing the *M. duvali* culture by another method would have produced a more viable suspension and a more effective vaccine against *M. leprae*. It is widely accepted that aqueous suspensions of attenuated tubercle bacilli, such as BCG, lose effectiveness as vaccines when they are killed. Other mycobacteria might be killed in the tissues after injection and be eliminated before they can stimulate an immune response. Persistent viability in the tissues is apparently not an absolute requirement for immunogenicity, however. In two experiments we have found *M. leprae* in aqueous suspension not to lose vaccine effectiveness when killed (19; unpublished data); in that work, persisting lymph node enlargement has given evidence that the dead *M. leprae*, or its antigen, has persisted in tissues for long periods. Thus, although the reported antigenic relatedness of *M. duvali* to *M. leprae* was the basis for its selection for study, the degree of its immunogenicity is clearly also an important factor in determining its vaccine effectiveness.

The *M. duvali* experiment was highly instructive from a technical standpoint since it showed how counts of pooled tissues of vaccinated mice can give a misleading notion of the values from individual mice. Although the distribution of values among unvaccinated mice was such that an arithmetic mean was fairly representative, the distribution among vaccinated mice was sometimes decidedly skewed. Accordingly, our experimental design in vaccine experiments has been modified. The AFB are now counted in the tissues of individual mice. Eight mice are taken from each group in each harvest as before, and one harvest is made just at the onset of the plateau phase and another 90 days later. The latter harvest is included to rule out mere growth delay (caused, for example, by activity exerted entirely in the very early stages of infection) and to differentiate the more lasting immunity associated with the imposition of a lower plateau level. The number of control groups is reduced to two, one at the beginning of the experiment and one at the end. To provide security against losses by deaths, 30 mice are included in each vaccinated group, and a larger number is included in the controls so that the *M. leprae* growth curve can be followed at 28-day intervals. Differences between groups are analyzed by nonparametric methods since the AFB harvests from individual mice are not distributed normally. In the protocol we used earlier (16), counts were carried out on the pooled tissues of eight mice from each group, and there were as many control as vaccine groups. Experience had shown that the control groups did not differ from the arithmetic mean of all the control groups by more than 0.3 $\log_{10}$. Consequently, protection was not considered significant unless the count was reduced from the mean by more than this factor. This rule was not useful, however, in assessing the significance between two vaccinated groups, both of which showed more than 0.3 log of protection.

GVH reactions had no observable effect on pulmonary infections with *M. tuberculosis* or on foot pad infections with *M. marinum*. These two pathogens are found primarily in the extracellular position, so activation of macrophages, a prominent feature of GVH reactions (1), might not have so much influence on them as it would against an obligate intracellular parasite such as *M. leprae*. The effect against *M. leprae*, although detectable, was minimal, however. In the first experiment there was a definite delay in the appearance of growth of *M. leprae* but no diminution in the plateau level of *M. leprae* ultimately attained. The effect was observed with parental (C3) cells administered intraperitoneally and with C3 and allogenic (A) cells administered locally. In the second experi-

ment, a distinct effect was seen only with the allogenic cells injected locally. Presumably the injection of allogenic cells locally resulted in sensitization of the recipient's lymphocytes as well, and the two-way immune reactions (lymphocyte transfer reactions) interfered temporarily with the growth of *M. leprae*.

During GVH reactions, mice have been reported to have increased resistance to intravenous infection with *Diplococcus pneumoniae* (2), *Salmonella typhimurium* (1, 2), and *Listeria monocytogenes* (1). Humoral and immune responsiveness is frequently depressed during GVH reactions, however (1). In patients with leprosy, it has been recently stated that the repeated intravenous injection of allogenic peripheral leukocytes results in dramatic clinical and bacteriological improvement (13). The mechanism of this pronounced effect was suggested to be a short-term GVH reaction that occurred in lepromatous patients as a result of delay in immune rejection of the allogenic lymphocytes. Delayed rejection of allogenic skin grafts has been demonstrated in lepromatous patients (7). The effects here observed in mice with GVH reactions were distinct but minimal in one experiment and not demonstrable in the other experiment. The effects seen in mice with local injection of allogenic cells into an ongoing infection were more prominent, and they suggest that other means of producing local immune reactions, or perhaps the direct injection of lymphocyte mediators, might be explored as possible therapeutic measures.

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