Neonatally Thymectomized Lewis Rats Infected with Mycobacterium leprae: Response to Primary Infection, Secondary Challenge, and Large Inocula

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Several experiments were carried out to measure the ability of neonatally thymectomized Lewis rats (NTLR) to limit multiplication of Mycobacterium leprae. NTLR inoculated in one hind footpad with $10^7$ viable M. leprae and challenged in the other hind footpad with $5 \times 10^9$ organisms simultaneously or 120 or 180 days later permitted multiplication in both sites. By contrast, immunologically intact rats similarly inoculated did not permit multiplication from either inoculum. NTLR and immunologically normal BALB/c mice were equally susceptible to infection with M. leprae, in that multiplication occurred regularly in the footpads of both species when inoculated with a bacterial suspension diluted to provide five organisms per footpad. Finally, multiplication occurred when five viable M. leprae diluted with $10^5$ heat-killed organisms were inoculated into the footpads of NTLR. Although there was some evidence that NTLR are not completely immunosuppressed, NTLR appear to be capable of detecting much smaller proportions of viable M. leprae than can be detected by immunologically normal mice.

The self-limited infection of mouse footpads by Mycobacterium leprae (8) has been of inestimable value in the development of new chemotherapeutic agents and in assessing the rate at which M. leprae are killed during treatment of patients (9, 11). However, because the ceiling of multiplication in the footpads of immunologically intact mice and rats is about $2 \times 10^9$ M. leprae, only a limited number of organisms may be inoculated to demonstrate infectivity. If $10^6$ M. leprae are inoculated into the footpad of an intact mouse, no multiplication can be detected, regardless of the proportion of viable organisms in the inoculum (7).

In evaluating various drug regimens in clinical trials, skin biopsy specimens are obtained periodically during treatment, and no more than 5,000 to 10,000 organisms are inoculated into the footpads of intact mice, so that multiplication of the infective M. leprae may be recognized. Although patients with lepromatous leprosy may start treatment with as many as $10^{11}$ to $10^{12}$ viable M. leprae, they rarely begin treatment with more than 10% of their organisms infective for mice. During effective therapy, therefore, the loss of only the first 99 to 99.9% viable M. leprae can be detected if 20 mice are used for each inoculum (2). This means that as many as $10^6$ to $10^8$ viable M. leprae may remain and that the infection is by no means eradicated when viable organisms can no longer be detected. After this point, the killing of M. leprae may be demonstrated only by the laborious method of long-term follow-up and the potentially hazardous withdrawal of chemotherapy. Thus, the value of the mouse footpad technique in short-term clinical trials may have been exploited fully, and its limited sensitivity greatly diminishes its usefulness in long-term trials in patients with lepromatous leprosy.

Consequently, there is a need for an experimental animal highly susceptible to M. leprae infection that can be used during the course of leprosy chemotherapy to detect small numbers of surviving M. leprae in the presence of large numbers of killed M. leprae. Such an animal must have minimal immunological responsiveness so that large numbers of dead M. leprae will not act as an immunogen, preventing subsequent multiplication of the few viable organisms. We have found that the growth ceiling of M. leprae in neonatally thymectomized Lewis rats (NTLR) is 100 to 1,000 times higher than that in intact mice (4). In NTLR inoculated with 5,000 M. leprae, the peak of multiplication is reached about a year after inoculation, and organisms may continue to be infective for...
mice for 2 or more years. By contrast, in intact mice or rats inoculated with the same number of *M. leprae*, the peak of multiplication is reached 4 to 6 months after inoculation. After this time rapid killing of the organisms occurs, probably as a result of an immune response (10).

Although NTLR are highly immunosuppressed, these animals do not develop wasting disease and appear to have a normal life span, which suggests that immunosuppression is not complete. The ceiling for multiplication of *M. leprae* in NTLR is about $10^4$ per footpad (8); we did not know whether this ceiling was imposed by an immune response or by other factors. This report deals with the results of a series of experiments designed to determine the immunological status of NTLR, particularly with respect to their ability to mount an immune response to large numbers of either viable or heat-killed *M. leprae*, thus preventing the multiplication of small numbers of viable *M. leprae* inoculated simultaneously or at a later date.

**MATERIALS AND METHODS**

Pregnant inbred rats of the Wistar/Lewis strain were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. These animals were specific pathogen free with a defined microflora. Mice of the inbred BALB/c strain were obtained from our own colonies.

Thymectomy was carried out in all instances before the animals were 24 h old, in most instances between 5 and 16 h after birth. The method, in which the animals were anesthetized by hypothermia and the thymus was removed by blunt dissection, was identical to that described by us previously (3).

The *M. leprae* used in these experiments was of a strain originally isolated by Shepard and used subsequently for most of the experiments in these laboratories. The methods of inoculation, processing of footpads, and counting the *M. leprae* were those described by Shepard (8) and Shepard and McRae (12).

**RESULTS**

In our initial experiment we compared the ability of both NTLR and intact Lewis rats to permit multiplication of a small number of *M. leprae* after inoculation of large numbers of viable *M. leprae*. NTLR and immunologically intact rats were inoculated in the left hind footpad (LHF) with $10^2$ viable *M. leprae* that were obtained by harvest from the tails and footpads of NTLR that had been inoculated intravenously about 18 months earlier. The rats of this experiment were challenged in the right hind footpad (RHF) with $5 \times 10^3$ viable *M. leprae* either simultaneously or 120 or 180 days later. Subsequent harvests of *M. leprae* were made at intervals from both the LHF and RHF.

In no instance did the numbers of acid-fast bacilli (AFB) increase in the LHF of normal rats (Table 1). Instead, the AFB decreased by 4.9- to 17.1-fold over the observation period of 18 months. Further, the multiplication of *M. leprae* was almost totally suppressed, indicating that the large inoculum could immunize intact rats. In only two instances were any AFB found in the footpads challenged with $5 \times 10^3$ *M. leprae*; in both instances, the organisms were found in only one footpad from the groups of three that had been inoculated. Because the increase was less than 10-fold greater than the inoculum, the AFB found could have been some of those originally inoculated.

On the other hand, there was multiplication from the inoculum of $10^3$ *M. leprae* in the LHF of NTLR. Moreover, there was multiplication from the small inoculum in the RHF. In most instances, the increase of AFB was not as great in these animals as in the control NTLR that had received only $5 \times 10^3$ *M. leprae*. However, $3.93 \times 10^3$ organisms were harvested after 1 year from the RHF of the NTLR challenged 120 days after the initial inoculation, yielding an average doubling time of 27.8 days. This was only slightly longer than the average doubling time of 26 days in the control NTLR that had received only $5 \times 10^3$ AFB a year earlier.

Having demonstrated that NTLR were reasonably immunosuppressed, we carried out experiments designed to determine (i) the minimal number of *M. leprae* required to infect NTLR, and (ii) whether small numbers of viable *M. leprae* could be detected in the presence of large numbers of killed *M. leprae*, a situation that might exist during treatment of patients.

Organisms for the simultaneous titration of *M. leprae* in the footpads of BALB/c mice and NTLR were harvested from the footpads of BALB/c mice during the logarithmic multiplication. The concentration of the organisms in the harvested suspension was adjusted with Hanks basic salt solution to $5 \times 10^3$ *M. leprae* per 0.03 ml, and further serial 10-fold dilutions of this suspension were made in Hanks balanced salt solution. From $5 \times 10^3$ to $5 \times 10^{-1}$ *M. leprae* were inoculated into both hind footpads of groups of ten mice and four NTLR. At intervals beginning 4 months after inoculation, *M. leprae* were harvested from the footpads of the mice, and growth curves were constructed. The initial harvests of *M. leprae* from NTLR were carried out 1 year after inoculation and were continued at intervals up to 17 months after inoculation.
The largest shown pads of intact animals were less than those expected, and the number of M. leprae found in footpads by thymectomy was diminished four to six times in NTLR compared with that of the neonatally thymectomized rats. Inocula ranging from one to a million organisms per footpad were used in inoculating the intact animals. No M. leprae were isolated from any of the four rats inoculated with 5 x 10^1 organisms, whereas we found an average of 7.05 x 10^6 M. leprae per footpad in a pool of four footpads from mice sacrificed 354 days after inoculation with the same dosage. This was not necessarily an indication of greater susceptibility of the mice; rather, the results could be accounted for by the irregular infection known to be produced by very small inocula (6).

To determine if large numbers of dead M. leprae interfered with the multiplication of small numbers of viable organisms, serial dilutions of the same suspension of M. leprae used for the titration in mice and NTLR were further diluted with an equal volume of a suspension of heat-killed (75°C for 30 min) M. leprae containing 10^7 organisms per 0.015 ml. Each 0.03-ml volume inoculated into both hind footpads of groups of four NTLR delivered numbers of viable M. leprae ranging from 5 x 10^6 to 5 x 10^7 plus 10^6 heat-killed M. leprae per footpad. As was done with NTLR receiving viable M. leprae only, rats were sacrificed at intervals starting 1 year after inoculation to determine if multiplication had occurred. In all instances, AFB har-
vested from these rats were passaged to the footpads of normal BALB/c mice as a further test of viability and the ability of the organisms to multiply.

The third bar in each group of Fig. 1 illustrates that multiplication of *M. leprae* in the footpads of NTLR was unaffected by the simultaneous inoculation of heat-killed *M. leprae*. Because approximately a 10-fold increase occurred in the number of *M. leprae* found in the footpads of the NTLR inoculated with 10^7 heat-killed organisms and 5 x 10^9 to 5 x 10^10 viable *M. leprae*, it is clear that multiplication had occurred. In the rats that received 5 viable *M. leprae* plus 10^9 dead organisms, 7.37 x 10^5 AFB were harvested 523 days after inoculation; this was not different from the 5.62 x 10^9 organisms found in the footpads of the rat inoculated with 10^7 dead organisms 362 days earlier. However, there was an obvious difference in morphology of the AFB. The organisms harvested from the animal inoculated with 10^7 dead *M. leprae* were beaded and stained poorly. On the other hand, organisms harvested from animals infected with 5 viable and 10^7 dead *M. leprae* stained brightly and appeared to be more solid. Further, the latter organisms were infective for the mouse; 1.11 x 10^9 AFB per footpad were harvested 184 days after inoculation of passage mice with 5 x 10^9 organisms, indicating that the AFB harvested from NTLR that had been inoculated 18 months earlier with 5 viable and 10^7 heat-killed *M. leprae* were indeed viable. In no instance did heat-treated *M. leprae* multiply when they were passaged into mice or NTLR.

**DISCUSSION**

Neonatal thymectomy of Lewis rats produces severe impairment of the cellular immune responses to a number of antigens (1, 5). Further, we have shown earlier that footpad and testis infection of rats with *M. leprae* was greatly enhanced by neonatal thymectomy (4). However, because there appeared to be a definite ceiling of the multiplication, which rarely exceeded 10^8 to 3 x 10^8, we were uncertain as to the degree of immunological suppression in these animals and whether or not the presence of large numbers of *M. leprae* would interfere with multiplication of a small inoculum of *M. leprae*.

A major objective was to determine the quantitative differences between the response of normal rats and mice and NTLR to *M. leprae* infection. In simultaneous titrations, BALB/c mice and NTLR were found to be equally sensitive to *M. leprae* infection. Approximately five *M. leprae* were required to infect animals of both species. However, multiplication in the footpads of the NTLR was approximately 100 times greater than that in the mice. Further, when 10^7 heat-killed *M. leprae* were used to dilute smaller numbers of viable *M. leprae*, it was possible to detect multiplication of the five viable organisms. The intact Lewis rat re-
sponded to *M. leprae* infection in a manner similar to that of the intact mouse. Small numbers of organisms (5 × 10⁵) increased progressively to a maximum of 6 × 10⁶ AFB, whereas inocula approximately 10 times greater than the ceiling expected in the intact animals did not increase but actually decreased to the normal ceiling. Furthermore, the large inoculum was highly immunogenic in the intact rat and almost totally suppressed the growth of *M. leprae* in the contralateral footpad, irrespective of whether the challenge dose (5 × 10⁵) was given simultaneously or 120 or 180 days after the initial inoculation.

The results in NTLR clearly demonstrated that these animals were highly immunosuppressed but that this suppression probably was incomplete. The large (10⁷) inoculum gave rise to multiplication of *M. leprae* in these animals, and in no instance did it prevent multiplication from the small challenge inoculum in the contralateral footpad. The increase of the 10⁷ inoculum in NTLR was erratic, possibly because this large number of organisms was close to the ceiling of approximately 10⁶ *M. leprae* known to occur in the footpads of these animals; also it may have had some immunizing effect. In only two instances did the 10⁷ inoculum in NTLR give rise to multiplication that reached or exceeded 10⁸ *M. leprae* per footpad. In one group of NTLR inoculated 9 months earlier, the number of AFB averaged 3.54 × 10⁶ per pad; in another group inoculated 12 months earlier, the average number of AFB per footpad was 1.59 × 10⁶. These numbers were not significantly different from the ceiling of 7.85 × 10⁷ AFB reached 9 months after inoculation of NTLR with 5 × 10⁶ *M. leprae*.

We were particularly interested in the fate of the 5 × 10⁶ challenge inoculum in the RHF of NTLR that had been inoculated in the LHF with 10⁷ *M. leprae*. During the first 6 months after challenge in the groups in which NTLR were challenged either simultaneously or 120 days after the large inoculum, multiplication of *M. leprae* in the RHF apparently proceeded at a normal rate. Six months after challenge there was an average of 1.81 × 10⁸ and 3.09 × 10⁸ *M. leprae* per footpad, respectively, in NTLR challenged 0 and 120 days after initial inoculation with 10⁷ *M. leprae* in the contralateral footpads. NTLR that received 5 × 10⁶ *M. leprae* only had an average of 2.89 × 10⁶ *M. leprae* per footpad, whereas NTLR challenged with 5 × 10⁷ *M. leprae* 180 days after inoculation with 10⁷ *M. leprae* appeared to demonstrate some inhibition of growth of AFB from the challenge inoculum. The RHF of these animals averaged 1.54 × 10⁶ *M. leprae* per footpad, or 18.8-fold fewer AFB than NTLR that had received 5 × 10⁶ *M. leprae* only. By month 9 and continuing through month 12 after challenge, there was substantial evidence that some degree of inhibition of multiplication from the challenge inoculum occurred in all NTLR previously inoculated with 10⁷ *M. leprae*. In the animals challenged simultaneously with the 10⁷ inoculum, the maximal number of AFB recovered from the RHF was 6.12 × 10⁶, an increase of 1.22 × 10⁶-fold over the initial 5 × 10³ inoculum. However, this was 12.8-fold fewer AFB than the ceiling of 7.85 × 10⁷ AFB found in the control group that had received 5 × 10⁶ *M. leprae* only. In NTLR challenged 120 days after the large inoculum there appeared to be no inhibition of multiplication of the AFB in the RHF 1 year after inoculation. At that time, there was an average of 3.93 × 10⁷ AFB per footpad, or an increase of 7.86 × 10⁶-fold over the original inoculum of 5 × 10³. The results in NTLR challenged 180 days after the initial inoculum were similar to those in the animals challenged simultaneously with the large inoculum. The maximal numbers of *M. leprae* from the challenged footpads were obtained 9 months after inoculation. These averaged 5.15 × 10⁶ per footpad or 15.1-fold fewer than the maximal numbers harvested from NTLR controls inoculated with 5 × 10⁷ *M. leprae*.

Thus, although immunosuppression of NTLR was not complete, it was nevertheless of a very high order both when viewed by itself and when compared with the responses of intact rats. Among the intact rats, 27 were immunized with 10⁷ *M. leprae* in the LHF. Challenge of these animals in the RHF with 5 × 10³ *M. leprae* resulted in a very limited infection in only three footpads. No AFB could be detected in the remaining 24 footpads, indicating that the initial large dose of *M. leprae* had induced a virtually complete immunity in the intact rats. By contrast, in the corresponding groups of NTLR inoculated with 1 × 10⁷ *M. leprae*, all 24 footpads developed substantial *M. leprae* infections after challenge with 5 × 10⁷ organisms.

Although immunosuppression of NTLR is not complete, it is possible to inoculate 2,000 times as many *M. leprae* into their footpads than into those of the intact mouse or rat and still demonstrate that multiplication could occur. This is of greater practical significance than the lack of total immunosuppression of NTLR. Because approximately 5 viable *M. leprae* are required to infect a mouse or an NTLR, we can detect no fewer than 1 viable *M. leprae* per 10³ in intact mice and as few as 1 per 2 × 10⁶ in NTLR. Therefore, it appears that NTLR has not only a high degree of immunosuppression but also...
good potential as a highly sensitive host for the detection of small numbers of *M. leprae*.

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**LITERATURE CITED**


