Effect of Thymectomy and Antilymphocyte Serum on *Mycobacterium leprae* Infection in Mice

A. HOWARD FIELDSTEEL and SUZANNE GARTNER

Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025

Received for publication 20 May 1975

BALB/c mice thymectomized at 3 to 5 days of age were studied to determine if this procedure would result in enhanced susceptibility to infection with *Mycobacterium leprae* and, if so, whether or not administration of antilymphocyte serum would further increase this susceptibility. The plateau for growth in the footpads of intact mice occurred 4 months after inoculation, whereas in the thymectomized and thymectomized plus antilymphocyte serum-treated groups the plateau occurred between months 11 and 12 after inoculation. Thymectomy resulted in at least a 10-fold increase in the number of *M. leprae* found in the footpads. Antilymphocyte serum did not appear to further enhance the *M. leprae* infection in the thymectomized mice. Although growth of *M. leprae* in the testes of both intact and thymectomized mice was erratic, the number of organisms reached a higher ceiling in the thymectomized groups. *M. leprae* harvested from all groups was passaged into intact mice at various intervals after inoculation to test for viability. Viable *M. leprae* were found at all intervals tested including 22 months after infection in the intact mice, suggesting that a chronic infection occurred that probably lasted during the entire life of the animals.

Shepard has shown that a predictable and uniform infection with *Mycobacterium leprae* can be induced in the footpads of intact mice (12, 13). However, multiplication of the *M. leprae* was limited, and the viability of the organisms was not well maintained. Rees et al. (10, 11) have produced a more massive and prolonged infection in mice thymectomized as adults, providing these animals were also lethally irradiated (900 R) and reconstituted with syngeneic bone marrow. Gaugas (2), on the basis of a single observation, reported that antilymphocyte serum (ALS) given weekly for 7.5 months enhanced *M. leprae* infection in mice that were thymectomized as adults. When adult mice were only thymectomized, their susceptibility to infection apparently did not alter.

Although neonatal thymectomy alone is known to enhance viral oncogenesis in mice (4, 6), it has not been deemed feasible in the study of *M. leprae* infection in mice because neonatally thymectomized mice usually develop wasting disease and die between 2 and 3 months of age (8, 9). Therefore, these mice would not survive long enough to permit enhanced multiplication of *M. leprae*, which would not be detectable until at least 6 months after inoculation. However, when mice are thymectomized beyond 3 to 4 days after birth, wasting disease does not develop.

The present experiments were carried out to determine whether mice thymectomized at 3 to 5 days of age would become more susceptible to *M. leprae* infection and, if so, whether or not administration of ALS would further increase this susceptibility.

**MATERIALS AND METHODS**

**Test animals.** All animals used in these experiments were BALB/c mice from our own colony. Mice (3 to 5 days old) were anesthetized by total immersion in crushed ice for 2 to 3 min. Thymectomy was carried out with the aid of a dissection microscope at ×15 magnification. The sternum was removed, exposing the thymus, which was freed from its attachments in the anterior mediastinum and removed by blunt dissection. Inspection of the area was made at higher magnification to be certain no thymic tags remained. The incision was closed with two 6-0 silk sutures and sealed with collodion. Breathing recommenced after the mice were placed under a 60-W incandescent lamp for a few minutes. Then they were returned as a group to their mother. This procedure produced less than 10% mortality. Before inoculation with *M. leprae* at 6 to 18 weeks of age, the mice were distributed into five groups, each group containing 15 to 30 mice and approximately equal numbers of males and females of about the same age.

**M. leprae strain.** The *M. leprae* used in these experiments was the Shepard mouse-passaged human strain obtained from Louis Levy (U.S. Public Health Service Hospital, San Francisco, Calif.). The methods of inoculation, processing of footpads, and
counting the *M. leprae* were those described by Shepard (12) and Shepard and McRae (15). All mice were inoculated in both hind footpads with 7.6 × 10^6 *M. leprae*. In addition, the males were inoculated in each testis with 5 × 10^9 *M. leprae*. At least two mice from each group were killed at various intervals to determine the number of acid-fast bacilli (AFB) per inoculated organ (testes and/or footpads) after different treatment regimens. The *M. leprae* harvested from the footpads of each group at various time intervals were periodically passaged into both hind footpads of groups of five intact mice (about 5 × 10^9 *M. leprae* per footpad) to determine whether the organisms were still viable. Two of these mice were killed 6 months later, and the AFB were counted. An increase of at least 10-fold in the number of AFB per footpad was considered evidence of viability. If the increase was less than 10-fold, the remaining mice were killed 3 to 6 months later, and a count of AFB again was made.

**ALS.** The ALS was a highly potent serum obtained from A. L. Monaco (Department of Surgery, Harvard Medical School, Boston, Mass.). It was prepared in his laboratory by the method of Gray et al. (3). Mice were given ALS intraperitoneally (i.p.) in 0.2-ml doses weekly for either 2, 4, or 6 months starting 3 months after inoculation of *M. leprae*. The mice were given two doses of 0.5 ml each of normal rabbit serum, which was administered i.p. 14 and 7 days before the start of ALS treatment to induce immune paralysis against the rabbit serum and prevent early immunity against the ALS (5).

**Calculation of generation time.** The average generation time (G) determined between time of inoculation and time of harvest, or the average time for *M. leprae* to undergo one doubling during this period, was calculated according to the formula used by Shepard and McRae (14): 
\[ G = t \log(H/F) \]
where \( t \) is the number of days after inoculation, \( H \) is the number of *M. leprae* harvested per footpad or testis, and \( F \) is the number of organisms contained in the inoculum.

(This work was present in part at the Eighth Joint Leprosy Research Conference, San Francisco, Calif., 30 July to 2 August 1973.)

**RESULTS**

The results of footpad inoculation are illustrated in Fig. 1 and summarized in Table 1. They show a normal growth curve for *M. leprae* in intact mice (group A). The maximum number of AFB in that group, 1.65 × 10^8 per footpad, was found 4 months after inoculation, and the numbers remained fairly constant through 22 months of observation. By contrast, growth of *M. leprae* in the other groups continued for 12 to 15 months after inoculation. The greatest number of AFB, 2.86 × 10^7 per footpad, was found 15 months after inoculation in the footpads of thymectomized mice that had received ALS for 4 months (group D). However, no significant differences were evident among the thymectomized ALS-treated groups, and the numbers of *M. leprae* encountered in the ALS-treated mice probably were not significantly greater than the numbers of *M. leprae* obtained from the footpads of mice that were only thymectomized. The basic difference between the intact and thymectomized groups is that at 11, 12, and 15 months, three periods when all groups were sampled, the average number of *M. leprae* from all 12 samples of the thymectomized groups was 1.34 × 10^7 per footpad, whereas the three samples from the nonthymectomized mice averaged only 1.03 × 10^6 per footpad.

At various intervals, beginning 11 months after inoculation, *M. leprae* harvested from the different groups were passaged into the hind footpads of intact mice to determine their viability. No attempt was made to determine the time to plateau or the death phase. *M. leprae* were harvested from the footpads 6 months after inoculation. Thus, it was possible to deter-
mine the relative viability of *M. leprae* harvested from the different groups and passaged at a particular time by determining the fold increase of the harvest over the inoculum. The 11-month passage from the footpads of group A mice showed no increase at a harvest performed 6 months after passage; only $5.05 \times 10^3$ AFB were recovered, as compared with the inoculum of $7.50 \times 10^3$. However, another harvest, made 9 months after inoculation, showed a 64-fold increase, giving a $G$ of 46 days. This indicated the presence of only a relatively few viable AFB in the inoculum from the intact mice, since Shepard and McRae (14) have shown the $G$ of *M. leprae* during the logarithmic phase of growth to be 12 to 13 days. AFB from all thymectomized groups showed a considerable increase in the passage mice when harvested 6 months after inoculation. The inocula from groups B, C, D, and E increased 69-, 31-, 47-, and 53-fold, respectively. The $G$ of the AFB from these groups were 28.9, 35.6, 31.9, and 30.9, respectively.

Fifteen months after inoculation, when the *M. leprae* from all groups were again passaged to the footpads of intact mice, a 105-fold increase occurred in the inoculum from group A mice, representing a $G$ of 28.2 days. The inocula from groups B, C, D, and E (harvested at 15 months) increased in the passage mice 23-, 140-, 68-, and 33-fold, respectively, representing $G$ values of 42.1, 26.4, 30.8, and 37.2.

When 10 remaining intact mice in group A were killed 22 months after inoculation, the pooled footpads each averaged $1.1 \times 10^6$ AFB. It is of interest to compare the viability of this harvest with that of harvests obtained after 11 and 15 months in the same group, because the number of AFB per footpad in all three instances was almost identical. The harvests at 11 and 22 months consisted of AFB that contained approximately the same proportion of viable organisms; the AFB from the 11-month harvest had a $G$ of 46 days, whereas the AFB from the 22-month harvest had a $G$ of 47.6 days. By contrast, the 15-month harvest, although it had virtually the same number of *M. leprae* as the other two groups, consisted of a much greater proportion of viable AFB as evidenced by their 28.2-day $G$. This was further evidence that, when the plateau phase was reached (4 months after inoculation of the intact mice), the infection not only did not remain static, but the mice were unable to mount an immune response capable of destroying the infectivity of the *M. leprae*.

Overall results of inoculating *M. leprae* into the testes of the mice were rather disappointing, although, again, *M. leprae* grew better in the thymectomized groups than in the intact animals (Fig. 2). Organisms from testes of intact mice from group A were harvested and counted on four separate occasions between month 11 and 17 after inoculation. The greatest number of organisms, $5.2 \times 10^4$ per testis, was found 15 months after inoculation. This represented only a 10-fold increase over the inoculum and a $G$ of 142 days. By contrast, three harvests were made from group B mice between month 4 and 16 after inoculation, and one harvest (11 months after inoculation) contained $1.72 \times 10^6$ *M. leprae* per testis, representing a 344-fold increase over the inoculum and a $G$ of 40 days. The other two harvests contained only $4.92 \times 10^6$ and $1.91 \times 10^4$ *M. leprae* per testis. Five testicular harvests were made from group C between 5 and 16 months after inoculation. The harvest at 16 months, consisting of four testes,
Fig. 2. Effect of thymectomy and thymectomy plus ALS treatment on testis infection of BALB/c mice with M. leprae. Symbols: ○, Normal mice (group A); ●, mice thymectomized only (group B); △, mice thymectomized plus ALS for 2 months (group C); ▲, mice thymectomized plus ALS for 4 months (group D); □, mice thymectomized plus ALS for 6 months (group E). Thymectomy was performed at 3 to 5 days of age. ALS treatment was initiated 3 months after inoculation of M. leprae and was given i.p. weekly in 0.2-ml amounts.

averaged 1.92 × 10^7 per testis for an increase of 3,840-fold and a G of 39.4 days. However, two of the remaining harvests, at 6 and 11 months, contained no M. leprae. One harvest at 12 months contained 8.55 × 10^6 M. leprae per testis for an increase of 171-fold, and another harvest at 13 months contained 1.06 × 10^6 organisms per testis, an increase of only 21-fold. There were six harvests of testes from group D. The numbers varied from a twofold increase, 1.06 × 10^6 AFB at 8 months after inoculation, to an 1,150-fold increase of 5.73 × 10^8 AFB at 8 months (G = 33.1 days). There were four harvests of testes from group E, ranging from a low count of 4.65 × 10^4 AFB per testis 7 months after inoculation to a high of 1.69 × 10^6 AFB (G = 32.9 days) 9 months after infection.

DISCUSSION

Although Gaugas was able to demonstrate that ALS enhanced M. leprae infection in mice thymectomized as adults, he was unable to show that thymectomy alone enhanced the infection (2). In the experiments reported here, mice were thymectomized at 3 to 5 days of age to avoid the development of fatal wasting disease that is generally associated with thymectomy in the immediate neonatal period. In addition, groups of mice were given different regimens of ALS treatment after footpad inoculation with M. leprae. Although the differences between intact and thymectomized groups are not striking, they are nevertheless significant. The plateau for growth of M. leprae in the intact mice occurred 4 months after inoculation, whereas in the thymectomized and thymectomized plus ALS-treated groups the plateau occurred between month 11 and 12 after inoculation. Although the ALS-treated mice showed higher counts than the thymectomized mice not receiving ALS, the counts were not significantly higher; thus, ALS did not further enhance M. leprae infection. When all thymectomized groups are considered together, there is unquestionable enhancement of the infection over that in intact mice, especially if one considers the average number of AFB from month 11 after infection until the last sample was taken. In the intact group six counts involving 28 footpads were made between month 11 and 22. These averaged 1.09 × 10^6 AFB per footpad. Seventeen counts involving 68 footpads were made in all thymectomized and thymectomized plus ALS-treated groups between months 11 and 19 after infection. These averaged 1.22 × 10^5 per footpad. However, most significant was the observation that 52 of these footpads had counts ranging from 1 × 10^6 to 2.86 × 10^6, whereas the greatest number of M. leprae found in the footpads of intact mice at any period was only 1.65 × 10^6, and that was noted 4 months after inoculation. Possibly, had thymectomy been carried out in the immediate neonatal period, the intact and thymectomized groups would have shown a more striking difference, such as that seen in rats thymectomized in the immediate neonatal period (1). Nevertheless, mice thymectomized at 3 to 5 days of age appear to retain some degree of immunological incompetence, and thus they have an increased susceptibility to footpad infection with M. leprae.

The growth of M. leprae in the testes of both intact and thymectomized mice was quite erratic in that, frequently, either no M. leprae could be found in inoculated testes or else an insignificant increase occurred. However, it is still probable that thymectomy and thymectomy plus ALS were effective in increasing susceptibility of the testes to infection with M. leprae.
Perhaps the most significant observation was that in all groups at all intervals tested, up to 22 months after inoculation, viable organisms could be detected. This was not unexpected in the thymectomized and thymectomized plus ALS-treated groups because of their lower immunocompetence. Shepard and McRae (14) have shown that when the logarithmic phase of growth of \textit{M. leprae} ended approximately 4 to 6 months after inoculation, it was followed by a death phase and then a second growth phase. Levy (7) further showed that the time to plateau remained constant for passages made from 138 to 180 days after inoculation, but that during the next 4 weeks the time to plateau increased markedly, corresponding to a loss of 99% of the viable \textit{M. leprae}. The results obtained with the intact mice confirm both of these observations. The 4-month harvest of the intact mice (group A) was $1.65 \times 10^6$ per footpad with a $G$ of 15.3 days. This compares favorably with the doubling time of 12.5 days during logarithmic multiplication calculated by Shepard and McRae. If we account for an initial lag phase of at least two weeks, known to occur before multiplication, the $G$ of the 4-month harvest would be 13.5 days. Since no increase occurred in the number of organisms between month 4 and 22, the calculated $G$ time of the harvested \textit{M. leprae} would increase. If, during this plateau phase, the organisms remained viable and did not multiply, we would expect that when they were passed to the footpads of other mice the doubling times in these mice would be similar to each other whether the organisms were obtained 11 or 22 months after inoculation of the original animals. This did not occur. When the AFB from the 11-month harvest (which had a $G$ of 47 days) were passed, the AFB from the passage mice had a $G$ of 46 days. This confirmed that the harvest made 11 months after inoculation contained few viable AFB, and that viability declined steeply after the plateau phase was entered. As expected, the 15-month harvest consisting of $1.15 \times 10^8$ AFB had an increased $G$ (65 days). However, the organisms inoculated into the footpads of passage mice had a $G$ of only 28.2 days, or 17.8 days less than the organisms from the 11-month harvest. The same was true of the organisms from the 16-month harvest. The average \textit{M. leprae} per footpad from that harvest ($1.62 \times 10^9$) had a $G$ of 64.9 days. When organisms were inoculated into passage mice, their $G$ was 29.8 days, or 16.2 days less than the 11-month harvest. By month 22 after inoculation, another drop in viability occurred. These organisms had a $G$ of 93.9 days and, when inoculated into passage mice, their $G$ was 47.6 days, very similar to the 46 days found for the 11-month harvest but increased by 19.4 and 17.8 days, respectively, over the generation time of the 15- and 16-month harvests in the passage mice. Thus, we also were able to demonstrate that, after growth of \textit{M. leprae} reaches the plateau phase, alternate cycles of death and growth occur in the footpads of normal mice. Apparently, even in immunocompetent mice, \textit{M. leprae} may set up a chronic infection that lasts during the entire life of the animal.

**ACKNOWLEDGMENTS**

This work was supported by the U.S.-Japan Cooperative Medical Science Program administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases (Public Health Service grant AI-08417).

**LITERATURE CITED**