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Sciatic Nerve of Normal and T200x5R Swiss White Mice Fails to Support Multiplication of Intraneurally Injected *M. leprae*[1]

Vanaja P. Shetty, Papinder S. Matharu, and Noshir H. Antia[2]

Involvement of the peripheral nerve is the special and most important feature of leprosy in man. There are several issues concerning the formation of neural granuloma and the special relationship between *Mycobacterium leprae* and the peripheral glia that remain unclear for lack of a suitable animal model depicting the spectral disease seen in humans. With this objective in mind, we chose to work on a murine model wherein viable *M. leprae* were microinjected into the sciatic nerve bypassing the blood-nerve barrier in normal and immunosuppressed Swiss white (S/W) mice. A predominant epithelioid and a macrophage granuloma were readily obtained in normal and in thymectomized-irradiated (TR) mice, respectively ([2, 11]). Therefore, it was felt that this model might serve as a window for studying the neural pathogenesis in leprosy. In order to obtain further insight into the fate of *M. leprae* exposed to the intraneural milieu, in the present study the viability, fold increase and clearance, if any, of *M. leprae* injected intraneurally were assessed sequentially using semiquantitative measures.

MATERIALS AND METHODS

Animals. Random bred male and female Swiss white (S/W) mice maintained under standard laboratory condition were used.

Experimental design. A total of eight experiments were carried out, using either human- (freshly obtained nodule biopsy from untreated lepromatous patient) or armadillo-derived *M. leprae* (liver biopsy frozen at –70°C obtained from Dr. Eleanor Stores (WHO-THELEP) in groups of normal and TR (T200x5R) mice. The results thus obtained were compared.

Immunosuppression. Three- to four-week-old, S/W female mice were thymectomized and the whole body was irradiated with five doses of 200 rads each at biweekly intervals ([3]). Intraneural inoculation was carried out 2 weeks after the last dose of irradiation.

Preparation of *M. leprae* suspension. Tissues were homogenized and bacterial suspensions were prepared using the standard procedure ([13]). Bacterial suspensions thus obtained were used within 24 hr. The size of the inocula used ranged between 4 to 40×10^6 bacilli suspended in a constant volume of 10 µl of normal saline. The baseline viability of the *M. leprae* inocula used for the intraneural injection was determined at this point using the foot pads of normal S/W mice. For this purpose, inocula not exceeding 1×10^4 per foot pad were injected into both hind foot pads of 8–10 normal S/W mice. Harvests were carried out at months 6, 7, 8, and 12 postinoculation to record the fold increase and percentage take.

Intraneural injection. Mice were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium. Both right and left sciatic nerves were exposed aseptically from under the biceps femoris, lifted from the surrounding tissue, and the *M. leprae* suspension was injected into the endoneurial space using a glass micropipette attached to a Hamilton syringe through a ½″ canula. Formation of a bleb within the nerve at the site of injection was used as a marker for a successful transfer (60%–70%). The wound was closed using sterile suture.

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[2] V. O. Shetty, Ph.D., Senior Research Officer; P. S. Matharu, B.Sc.; N. H. Antia, F.R.C.S., F.A.C.S. (Hon.), Director and Trustee, The Foundation for Medical Research, 84-A R.G. Thadani Marg, Worli, Mumbai 400 018, India.

Reprint requests to V. P. Shetty, Ph.D., at the above address or FAX: 91-22-493-2876; e-mail: frchbom@bom2.vsnl.net.in

TABLE 1. *Average* M. leprae *yield per nerve × 104 ± S.D. (N = at least 6) at sequential intervals using normal (N) and T200x5r (TR) S/W mice (Expts. 1 to 8).*

ML source	Hamster		Armadillo		Human		Armadillo	
Mice	N	N	N	N	N	TR	TR	TR
Inoc. size	4×10^6	2×10^6	46×10^6	46×10^6	20×10^6	4×10^6	35×10^6	40×10^6
Biopsy interval								
24 hr	—	16.4 ± 8	6 ± 11	11 ± 12	8 ± 11	—	5 ± 8	21 ± 18
72 hr	—	—	46 ± 13	46 ± 28	16 ± 18	—	—	117 ± 77
1 wk	20[a]	—	3 ± 3	19 ± 25	4 ± 9	22[a]	19 ± 17	—
2 wks	30[a]	—	9 ± 11	32 ± 33	7 ± 64	33[a]	3 ± 2	37 ± 28
3 wks	—	—	16 ± 20	17 ± 10	5 ± 6	—	8 ± 7	—
4 wks	20[a]	3.4 ± 2	8 ± 8	10 ± 14	4 ± 5	40[a]	2 ± 2	83 ± 42
3 mos.	—	16.7 ± 4	30 ± 32	23 ± 32	4 ± 6	—	2 ± 1	50 ± 27
6 mos.	—	2.3 ± 0.5	—	44 ± 9	19 ± 22	—	24 ± 39	—
1 yr	—	0.2 ± 0.5	—	—	—	—	—	98 ± 40

[a] Pooled.

Biopsy. In experiments using armadillo *M. leprae*, the nerve biopsies were obtained at 24 hr, 72 hr, 1 week, and 2, 3, 4, 12, 24 and 48 weeks following intraneural injection of *M. leprae*. In experiments using human-derived *M. leprae*, biopsies were obtained only at select intervals for want of inocula (Table 1). A minimum of three mice (six nerves) were biopsied each time. Before biopsy, the mice were examined for clinical signs of hind limb weakness. Mice were sacrificed using ether anesthesia. The sciatic nerve was exposed surgically and changes such as adhesion, swelling and thickening, if any, at the site and along the length of the nerve were recorded. The whole length of the nerve was dissected out and transferred to a sterile vial containing a few drops of minimal essential medium (MEM) and stored in an ice bucket until homogenization. Both left and right hind foot pads, the liver, spleen, earlobes and the regional lymph nodes were also collected at regular intervals for homogenization to check for bacterial dissemination.

Quantitation of bacterial load in the nerve. Using aseptic measures, the biopsied sciatic nerves were cleaned using MEM containing penicillin to clear all of the epineurial and most of the perineurial tissues. Nerves thus cleaned individually were transferred to a 1-ml capacity glass homogenizer and homogenized using a fixed volume (0.25 ml) of normal saline per nerve. From the homogenate thus obtained, spot slides were prepared, fixed and stained using the Ziehl-Neelsen method. The bacterial load per nerve was determined.

Viability tests using mouse foot pad method. After determining the bacterial

TABLE 2. *Viability of* M. leprae *derived from intraneural experiments tested using mouse foot pads.*

Source of inoc.	TR mice Human ML		TR mice Armadillo ML		Normal mice Human ML		Normal Armadillo ML	
Biopsy interval	No. inoc.	No. positive takes[a]	No. inoc.	No. positive takes[a]	No. inoc.	No. positive takes[a]	No. inoc.	No. positive takes[a]
24 hr	—	—	2	6/26[b]	—	—	3	13/42
72 hr	—	—	1	4/14	—	—	3	6/34
1 wk	1	7/7	1	0/11	1	8/9	3	4/30
2 wks	1	8/9	2	7/23	1	5/8	3	8/33
3 wks	—	—	1	1/10	—	—	3	5/35
4 wks	1	4/6	2	3/26	2	7/16	3	7/36
3 mos.	—	—	2	0/14	1	0/9	3	5/32
6 mos.	—	—	1	0/11	1	0/11	1	0/4
1 yr.	—	—	1	0/8	1	0/10	1	0/10

[a] Fold increase ≥10.
[b] Denominator is the number of harvests.

TABLE 3. M. leprae *yield per nerve* $\times 10^6$ *at zero and 24 hr in the endoneural and epineural compartments (Expts. 9 and 10).*

ML source	Armadillo				Armadillo			
Mice	Normal S/W				Normal S/W			
Inoc. size	20×10^6				40×10^6			
Biopsy interval	0 hr		24 hr		0 hr		24 hr	
No.	Endo.	Epi.	Endo.	Epi.	Endo.	Epi.	Endo.	Epi.
1	1	0.2	0.6	0.2	4	0.02	4.8	0.1
2	2.3	0.05	2.7	0.07	1.0	0.04	1.6	4.1
3	2.5	0.2	2.6	0.37	2.7	1.0	0.5	0.02
4	0.3	0.01	0.4	1.9	6.6	0	4.0	0.2
5	0.8	2.2	0.6	1.0	0.03	1.6	3.6	0.1
6	4.7	0.07	0.4	0	1.0	0.8	2.7	0.2
Mean ± S.D.	1.9 ± 1.6	0.09 ± 0.08	1.4 ± 1.1	0.7 ± 0.7	2.6 ± 2.4	0.6 ± 0.7	2.9 ± 1.6	0.8 ± 1.6

load per nerve, the nerve homogenates were pooled for each interval and injected into the foot pads of normal S/W mice with an inoculum not exceeding 1×10^4 *M. leprae*/foot pad. The foot pad harvests were carried out at months 6, 7, 8, and/or 12 postinoculation (Table 2).

Determination of loss of *M. leprae* between zero and 24 hr. In order to determine the extent of loss of *M. leprae*, between zero and 24 hr after intraneural injection, two separate experiments were carried out (Table 3) using armadillo *M. leprae*. In these experiments endoneural and epineurial tissues were separately and individually harvested in order to determine the seepage, into the epineural compartment during the injection.

In order to check the spread of *M. leprae* along the length of the nerve, in two of the experiments (in Table 1 Nos. 5 and 8) the whole length of the nerve was divided into three segments, i.e., a) the middle 2-cm length to include the site of injection; b) the proximal and c) the distal parts of the nerve were separately homogenized and counts were taken.

RESULTS

***M. leprae* yield/nerve at sequential intervals following intraneural injection.** A total of 210 counts per nerve were obtained at sequential intervals beginning 24 hrs, 72 hrs, 1, 2, 3, and 4 weeks, and at 3, 6, and 12 months. The over-all results (Table 1) show that approximately 1 out of 10 of the total inocula was recovered from the endoneural compartment of the nerves at 24 hr. Further, for reasons not very clear, the average recovery of *M. leprae* per nerve at 72 hr was 10 times higher. At the subsequent intervals until month 6, the counts were comparable to the 24-hr yield per nerve. Most importantly, there was neither a significant increase nor a decrease in the *M. leprae* yield per nerve in any of the experiments carried out, using either normal or T200x5R mice.

Baseline viability of *M. leprae* used in intraneural experiments. Both human-derived *M. leprae* (nodule biopsies from two untreated lepromatous patients) inoculations into the foot pads of normal S/W mice gave a maximal fold increase of 10^6 per foot pad and the take was 100% (results not shown).

The armadillo-derived *M. leprae* used in the remaining two experiments were similarly tested at three intervals, once on arrival and once each time before use in the first and the last experiments. On arrival, maximal yield was 10^6 per foot pad and take was 100%, like that of the human-derived *M. leprae*. The viability test carried out the second and third time, i.e., after 2 and 2½ years of storage at –70°C, showed a maximal fold increase of 10^5 at month 12; the take was 100%, but the data suggest that there was a decline in viability on prolonged storage.

Viability of *M. leprae* derived from intraneural experiments. In order to determine the viability of *M. leprae* exposed to the intraneural milieu for different lengths of time (24 hr to 12 months), a total of 46 such pooled inocula obtained at sequential intervals from experiments 1 to 8 were

tested using the foot pads of normal mice. The foot pad harvests were carried out 6, 7, 8, and/or 12 months postinoculation. Viable bacteria were detected only in the inocula that were obtained between 24 hr and 4 weeks. However, as compared to baseline, all inocula obtained from the intraneural experiments gave a fold increase that was less than baseline values by 10-fold. Further, only a few counts reached the ceiling of 10^5 in the experiments using armadillo *M. leprae.* The results obtained with human-derived *M. leprae* were comparatively better, both in terms of percentage take as well as fold increase. Beyond 4 weeks, however, there was a sharp decline, and a total loss of viability was noted by 12 months with inocula obtained from both normal and TR mice using human- as well as armadillo-derived *M. leprae.*

In the present experiment there was no significant loss of the inocula between zero and 24 hr as ascertained in two separate sets of experiments.

No detectable *M. leprae* were obtained in other tissues tested except in the regional lymph nodes in which small numbers of bacilli were obtained during the early intervals (results not shown). No significant *M. leprae* counts were obtained in either the proximal or the distal segments, thus excluding any spread along the length of the nerve up to 12 months after inoculation. Therefore, it was concluded that the *M. leprae* inocula remained highly contained.

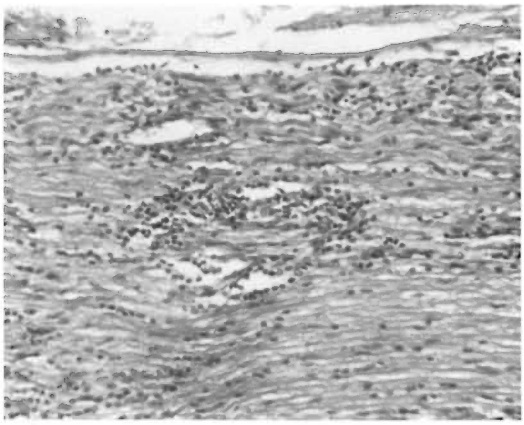

Fig. 1. Longitudinal section of a 3-month-old sciatic nerve lesion obtained from T200x5R mouse injected intraneurally with 20×10^6 armadillo-derived *M. leprae.* Note presence of dense perivascular (arrow) and scattered lymphocytes in the endoneurium (paraffin-embedded tissue section stained with TRIFF ×650).

DISCUSSION

In the present study, the fate of *M. leprae* injected into the sciatic nerve in normal and TR mice was investigated using semiquantitative methods. The over-all results show that the freshly harvested *M. leprae* that were injected into the sciatic nerve remained within the nerve in a highly contained manner. While the number of *M. leprae* remained by and large unchanged between 24 hr and 6 months, viable bacteria were detected only until 4 weeks after inoculation. Thus, the intraneural compartment failed to sustain the viability and support the multiplication of *M. leprae* in either the normal or the TR mice under these experimental conditions.

The intraneural route of inoculation has its limitations. First and foremost, a successful inoculation could be achieved in only 60% to 70% of the cases even with experience. Secondly, less than one-tenth of the inoculum could be recovered from the endoneurium at 24 hr. Seepage of part of the inoculum into the epineurial compartment was unavoidable which, in turn, resulted in wider variations in the *M. leprae* yield per nerve. Despite these limitations certain observations deserve further discussion.

There were potentially instructive differences between the present intraneural

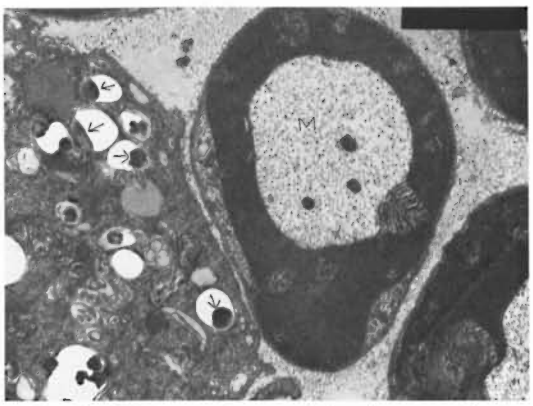

Fig. 2. Electron micrograph showing part of sciatic nerve obtained 72 hr after intraneural injection with human-derived *M. leprae* in a normal mouse. Note presence of several bacilli in membrane-bound vacuoles (arrow) within the ctoplasm of a macrophage. A well-preserved myelinated fiber (M) is seen in close proximity (×8500).

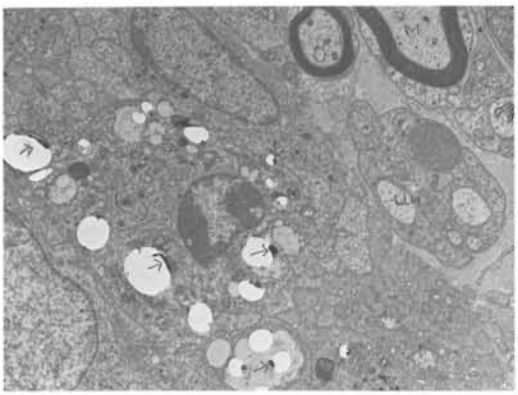

Fig. 3. Electron micrograph showing a 9-month-old sciatic nerve lesion in a TR mouse. Note presence of a large foamy macrophage carrying several bacteria in membrane-bound vacoules (arrow). M = thinly myelinated fiber; Un = nonmyelinated fiber complex (×4000).

model and the well-established mouse foot pad model ([10]). Contrary to our expectation, seeding of *M. leprae* into the endoneural compartment, and thus bypassing the blood-nerve barrier, failed to allow the bacilli to retain their viability and failed to support their multiplication even in T200x5R mice. This was unlike the foot pad where 100- and 1000-fold increases are normally obtained by months 12 and 16 postinoculation in normal and T200x5R mice, respectively ([3]). With the intraneural route of infection, the specific granulomatous reaction peaked at 3 to 4 weeks ([2, 11]), and this coincides with further loss of viability of *M. leprae*. The loss in viability did not show any relevance to the type of infiltrating cells. Unlike this, following foot pad inoculation of *M. leprae* appreciable granulomatous reaction at the site occurs much later, i.e., 2 to 2½ years after the foot pad inoculation ([7, 10]). In the foot pad model, distinct and reproducible pathological changes occur in the sciatic nerves in mouse strains such as CBA, C57 and S/W ([7, 12]). Contrary to earlier claims ([7]), however, *M. leprae* and granulomatous infiltrate were conspicuously absent in the nerve lesions that were followed up for 2½ years in our laboratory using the C57 and S/W strains of mice ([12]).

With the intraneural route of infection in mice there was a specific inflammatory response (Fig. 1) and ensuing damage to nerve fibers, but *M. leprae* were not traced to the Schwann cells (Figs. 2 and 3) during the 1-year course of the study. This is unlike the well-documented findings *in vitro* in which *M. leprae* were readily phagocytized by both human and murine Schwann cells ([6, 8]). Several well-documented studies in armadillos show that the entry of *M. leprae* into the nerves is highly restricted despite a disseminated infection ([4, 9, 14]). This was also the observation in nude mice ([5]). In armadillos, it was noted that the average time taken to develop disseminated infection was 12 to 29 months. Animals given a low dose of bacilli, i.e., 1×10^3, intradermally developed infection sooner than infected with a larger dose, i.e., 3.5×10^7. It was thought that the larger dose might have sensitized the animals and delayed the development of generalized infection. There was no significant difference otherwise in the susceptibility of the animals related to the routes used, i.e., intravenous and subcutaneous, or the dose of infection ([4]). A more recent ultrastructural study reports a striking preponderance of *M. leprae* in the endothelial cells around the nerve, i.e., epineurium and perineurium ([9]). This and several other manifestations of the disease in the armadillo are unlike that in humans. The early response of traumatized rabbit tibial nerve to epineurial infection with *M. leprae* revealed phagocytosis of *M. leprae* by the macrophages at the site. There was no further follow up ([2]). To our knowledge, there is no documented long-term study using the intraneural route of infection with viable *M. leprae* in animals.

In conclusion, the peripheral nerve compartment in both normal and T200x5R mice failed to support the multiplication of *M. leprae*. There was a highly effective localization and containment of *M. leprae* infection within the endoneural compartment. Therefore, the peripheral nerve in S/W mice was neither a favored nor a protected site for *M. leprae* multiplication under these experimental conditions. Is this related to the difference in temperature at the site or could it be a reflection of a species-related difference in tissue tropism? In our opinion, there are several indicators that favor the latter.

SUMMARY

In a preliminary study we have shown that freshly harvested *Mycobacterium lep-*

rae, when injected into the sciatic nerve in normal and immunosuppressed (TR) mice, induce massive but localized epithelioid and macrophage granuloma, respectively, in 3–4 weeks. In order to determine the fate of *M. leprae* injected intraneurally into normal and TR mice, in the present study we measured sequentially the viability, fold increase and clearance, if any, using semiquantitative methods. The average *M. leprae* yield per nerve assessed at regular intervals, beginning at 24 hr and including 72 hr, 1 week, 2, 3, 4, 12, 24 and 48 weeks, showed neither a significant increase nor a decrease in either the normal or the TR mice. The viability of *M. leprae,* assessed using the standard mouse foot pad method, showed a significant decrease as compared to baseline growth effective at 24 hr and remained static until approximately 4 weeks. A further decline and total loss of viability was noted by 12 months. The results show that injection of *M. leprae* via the intraneural route in both normal and TR mice failed to sustain the viability and failed to support the multiplication of the organisms.

RESUMEN

En un estudio preliminar demostramos que *Mycobacterium leprae* recién aislado, inyectado en el nervio ciático de ratones, induce, en 3–4 semanas, granulomas epitelioides en ratones normales y granulomas macrofágico en animales inmunosprimidos (TR). Para determinar el destino de los bacilos en estos animales, en el presente estudio medimos secuencial- y semicuantitativamente la viabilidad, la multiplicación y la depuración de los bacilos. El número promedio de bacilos por nervio a las 24 h, 72 h, 1 semana, 2, 3, 4, 12, 24 y 48 semanas, no mostró cambios ni en los ratones normales ni en los ratones TR. La viabilidad de *M. leprae,* según el método etsándar de la amohadilla plantar del ratón, se encontró significativamente disminuida en comparación con la viabilidad del bacilo observada a las 24 h de inoculación y permaneció estática hasta aproximadamente 4 semanas. Después, a los 12 meses se observó una posterior declinación y la pérdida total de la viabilidad. Los resultados muestran que la inyección de *M. leprae* via la ruta intraneural en ratones normales y en ratones inmunosuprimidos, no sostiene ni la viabilidad ni la multiplicación del microorganismo.

RÉSUMÉ

Dans une étude préliminaire, nous avons montré que des *Mycobacterium leprae* récemment récoltées et injectées dans le nerf sciatique de souris normales ou immunosupprimées (TR) provoquent la formation massive mais locale de granulômes macrophagiques de type épithélioïdes ou histiocytaires, respectivement, en 3 ou 4 semaines. Dans cette étude, afin de déterminer le devenir de *M. leprae* après une injection intraneuronale de *M. leprae* chez des souris normales ou TR, nous avons mesuré, de façon séquentielle et si cela était approprié, la viabilité, les facteurs de croissance et d'élimination par des méthodes semi-quantitatives. Le rendement moyen de *M. leprae* par nerf, évalué à des intervalles de temps réguliers, débutant à 24 heures et incluant 72 heures, 1 semaine, 2, 3, 4, 12, 24, et 48 semaines n'a révélé ni d'augmentation ni de diminution significative que les souris fürent TR ou normales. La viabilité de *M. leprae,* évaluée par la méthode standard d'inoculation dans la patte de souris, a montré une diminution significative dès 24 heures, comparée aux valeurs de croissance basales avant inoculation. Cette diminution resta constante jusqu'à approximativement 4 semaines, puis un second déclin et une perte totale de viabilité furent notés autour de 12 mois. Ces résultats indiquent que l'injection intra-neurale de *M. leprae* ne permet pas la survie et ne soutient pas la multiplication de cet organisme chez les souris normales ou immunosupprimées (TR).

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