Animal Model to Study the Mechanism of Nerve Damage in Leprosy—A Preliminary Report[1]

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Since 1881 over 30 animal species have been tried for the experimental induction of leprosy, and today we are using a few strains of rats ([6]) and mice ([17]), nude mice ([8]), three species of armadillos ([19]), the Korean chipmunk ([9]), and four nonhuman primates ([10]) in this successful list. Nerve lesions are documented in all of the animal models with minor variations related to route of infection and with susceptibility/resistance factors playing a role ([7]). A major limitation has been that none of the animal models really depict the spectral disease of human leprosy, and the slow progressive nerve damage seen in these models is representative, at best, of borderline disease. Thus, the search is still on ([4]) for a better animal model to use in studying nerve damage during the normal course of the leprosy infection and during the various reactional states.

Our present study aims at developing an animal model for studying the kinetics of nerve damage induced by the tuberculoid and lepromatous types of granulomatous responses and also the effect of selective deletion of macrophages on the granulomatous response and nerve damage in a time-bound manner. For obvious reasons such studies cannot be undertaken on naturally occurring lesions.

In a preliminary study we have used intraneural injection of *Mycobacterium leprae*, a technique followed by Wisniewski and Bloom ([18]) to bypass the blood-nerve barrier. Using this technique Mshana, *et al.* ([11]) and Cowley, *et al.* ([3]) have induced granulomatous responses within the sciatic nerves of rabbits and guinea pigs presensitized to mycobacterial antigens.

Unlike these researchers, we choose to use viable *M. leprae* in unsensitized, normal and immunomodulated mice since there is evidence suggesting that the molecular level response to viable *M. leprae* may be different from that of nonviable *M. leprae,* particularly in the neural environment ([13]). The results obtained were encouraging and are the subject of this report.

MATERIALS AND METHODS

A total of 20 right and left sciatic nerve biopsies obtained from ten mice were studied in two sets of experiments using a protocol as shown in The Table. Normal, immunosuppressed (T200 × 5R) and silica-treated, Swiss white female mice were injected intraneurally with $10-20 \times 10^6$ viable *M. leprae* suspended in normal saline.

In experiment 1, the *M. leprae* used for intraneural injection were obtained from a fresh nodule biopsy of an untreated lepromatous patient. In experiment 2, *M. leprae* were obtained from a frozen armadillo liver. The tissues were homogenized using the technique of Shepard ([17]). The bacterial suspension thus obtained was used for intraneural injection within 24 hr.

Immunosuppression of mice. Three-to-four-week-old, Swiss white female mice were thymectomized and irradiated with five graded doses of 200 rads each × irradiation at biweekly intervals (T200 × 5R) ([2]). These mice were used for intraneural injection 2 weeks after the last dose of irradiation.

Treatment of mice with silica for macrophage inactivation. Adult, Swiss white female mice were given intraperitoneal injections of 1 ml of 20% silica quartz dust (size 80% of particles, $1-5 \mu$m; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) suspension in 0.5% buffered saline solution (BSS), 1 day prior to intraneural injection of *M. leprae* and/or a crush injury to the sciatic nerve. The silica dose was repeated two times per week thereafter until biopsy. Treatment with silica quartz dust selectively inhibits macrophage function *in situ* ([1]). Thus, in the present study, normal mice with a crush

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THE TABLE. *Number of mouse sciatic nerves studied using different experimental protocols.*[a]

Experiment	Source of *M. leprae*	(1) N + ML	(2) TR + ML	(3) N + Si + ML	(4) N + Si + crush + ML	(5) N + Si + crush	(6) N + crush	Total
1	Human nodule	2	2	—	2	2	2	10
2	Armadillo liver	2	2	2	2	2	—	10
							Total	20

[a] N = Normal mice; TR = thymectomy and irradiation (T200 × 5R); Si = silica-treated mice; ML = *M. leprae.*

injury to the sciatic nerve (standard model for Wallerian degeneration) and mice treated with silica (+Si) followed by a crush injury to the sciatic nerve were used as controls to assess the effect of *M. leprae* in the nerve with or without a crush injury in silica-treated mice (protocol as in The Table).

Intraneural injection procedure. Mice were anesthetized with an intraperitoneal injection of 1% pentabarbitone. Using a dissecting microscope, both the right and left sciatic nerves were exposed aseptically from under the biceps femoris, lifted from the surrounding tissue, and 0.5 μl of *M. leprae* suspension containing $10-20 \times 10^6$ viable *M. leprae* were injected into the endoneurial space using a 30-gauge needle and a Hamilton syringe. A cyclomixer was used to get an even bacterial suspension.

For inducing a crush injury, a fine watchmaker's forcep was used. In group 4 (The Table) the nerve was first crushed, then immediately injected with the *M. leprae* suspension, and the wound was sutured.

Biopsy. The sciatic nerve biopsies from all of the mice were obtained using pentabarbitone anesthesia 2 weeks after the intraneural injection of *M. leprae* and/or a crush injury. The nerves were fixed in 2.5% glutaraldehyde, postfixed in osmium, dehydrated, and embedded in araldite. One-μm-thick, semithin sections were stained with toludine blue and studied under the light microscope. Subsequent ultrathin sections stained with uranyl acetate and lead citrate were studied using a JEOL 100S transmission electron microscope.

RESULTS

Normal mice with intraneural *M. leprae*. One-μm-thick resin sections stained with toludine blue revealed patchy areas of fiber loss. There were cuffs of lymphocytes around the dilated blood vessels, and a few aggregates of epithelioid cells and giant cells were also seen (Fig. 1A).

Scanning the sections under the electron microscope confirmed the light-microscopic findings (Fig. 1B). A carpet of epithelioid cells and a few giant cells were seen in the endoneurium. Occasional macrophages were seen with bacilli, single or in small clusters. No bacilli were traced to Schwann cells. Myelin debris in various stages of disintegration was seen mostly in the macrophages. Schwann cells appeared active and increased in number. A few regenerating fibers were also seen. The lesions seen in these nerves compared well with the human tuberculoid type of lesions as per the Ridley-Jopling classification ([15]).

Immunosuppressed (T200 x 5R) mice with intraneural *M. leprae*. There was a more diffuse and moderate loss of nerve fibers. There was only macrophage infiltrate in these nerves. Occasional lymphocytes were seen, but the epithelioid type of macrophages or giant cells were not seen. The rest of the changes seen were similar to normal mice with intraneural *M. leprae*. Here, again, *M. leprae* were seen only in the macrophages.

Silica-treated mice with a crush injury. There was no macrophage infiltrate in these nerves. Fairly intact myelin rings were seen around collapsed and degenerated axons within the Schwann tubes. No activation or proliferation of Schwann cells nor any regeneration or remyelinating activity was seen in these nerves (Fig. 2A and 2B).

In contrast, nerves with only a crush injury (group 6) revealed macrophage infiltrate, phagocytosis, and a breakdown of myelin by the macrophages. There was
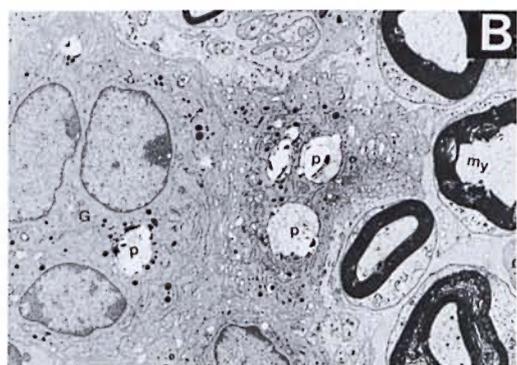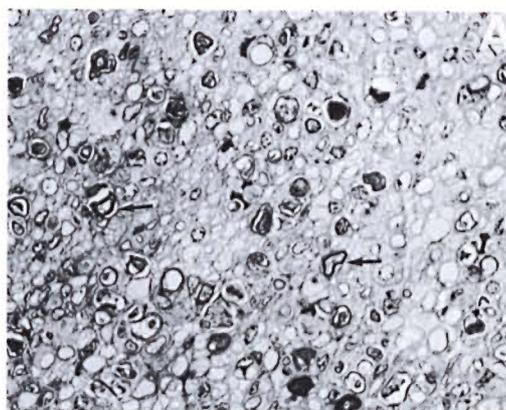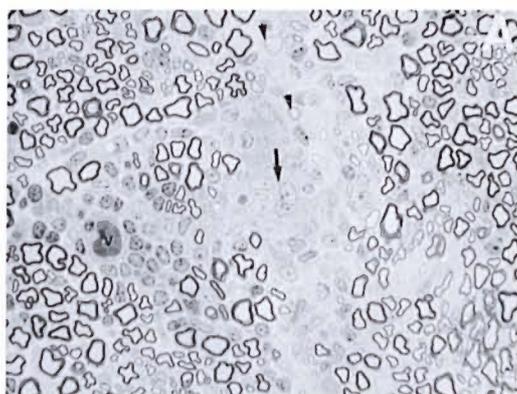
FIG. 1.   **A.** Part of the sciatic nerve from a normal mouse injected intraneurally with viable *M. leprae* suspension 2 weeks prior to biopsy showing patchy areas of fiber loss. Note the presence of lymphocytic infiltrate around one of the blood vessels (v). Several pale, nucleated epithelioid cells and one giant cell are also seen (arrow). A few thinly myelinated regenerative fibers are seen (arrowhead) on an edematous background. This lesion is comparable to an early human tuberculoid type of nerve lesion (semithin transverse section stained with toludine blue ×750). **B.** Electron photomicrograph of a part of the Figure 1A nerve showing one multinucleated giant cell (G) surrounded by a few epithelioid macrophages in the endoneurial area. A few phagosomes (P) with bacterial debris are also seen within these cells. My = myelinated fiber (uranyl acetate plus lead citrate stain ×6000.)

FIG. 2.   **A.** Part of the sciatic nerve with a crush injury in a silica-treated mouse from a biopsy obtained 2 weeks after a crush injury. No normal-looking myelinated fibers, macrophages, or activation of Schwann cells are seen. Regeneration and remyelinating fibers are also not seen in this nerve. Note the presence of large numbers of darkly stained myelin rings (arrow) (semithin transverse section stained with toludine blue ×750). **B.** Electron photomicrograph of a part of the crushed nerve from the silica-treated mouse in Figure 2A. Note the degenerate and collapsed axons (a) and fairly intact myelin rings lying within the Schwann cells (S). Some membraneous inclusions are also seen in the Schwann cell cytoplasm. Note the absence of macrophages and regenerative activity in this nerve (uranyl acetate plus lead citrate stain ×5000).

Schwannian proliferation and regeneration of fibers, suggesting that in silica-treated mice the normal process of degeneration and regeneration following a crush injury was retarded in the absence of macrophages.

Silica-treated mice with/without a crush injury and *M. leprae*. When *M. leprae* were injected along with a crush injury in the silica-treated mice (group 4), the lesion obtained was very similar to that seen with a crush alone, although the extent of the macrophage infiltrate was less. Myelin degradation was not as effective as in the mice without silica but regenerative and remyelinative activity was very good (Fig. 3). The epithelioid type of macrophages and lymphocytes were not seen in these nerves, and bacilli were seen only in the macrophages.

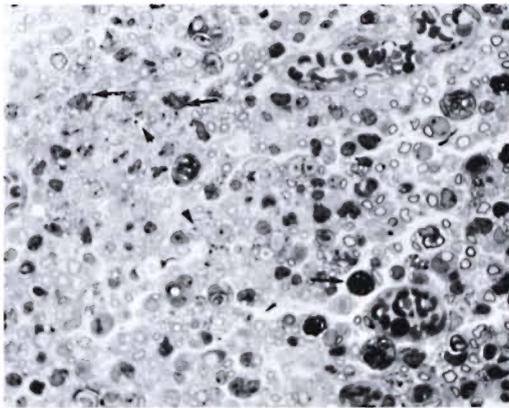When *M. leprae* were injected intraneurally without crushing the nerve in silica-

FIG. 3. Part of the sciatic nerve with a crush injury plus intraneural *M. leprae* in a silica-treated mouse. There are uniformly distributed, small, thinly myelinated fibers suggesting regeneration. Most of the darkly stained structures (arrow) are the myelin debris, some within macrophages and some within Schwann cells. Note the presence of large numbers of bacilli within some of the macrophages (arrowhead) (semithin transverse section stained with toludine blue ×750).
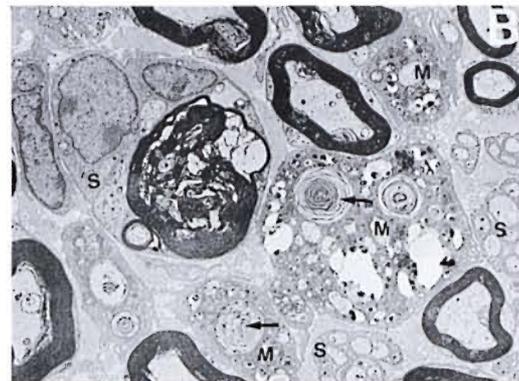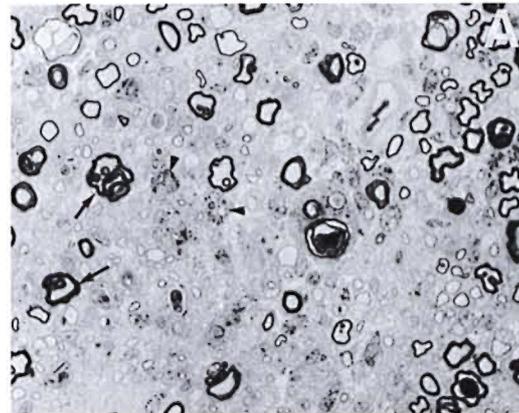

FIG. 4. A. Part of the sciatic nerve injected intraneurally with viable *M. leprae* in a silica-treated mouse from a biopsy obtained 2 weeks after intraneural injection of *M. leprae*. Some of the myelinated fibers show extensive folding of the myelin (arrow), and there are several thinly myelinated regenerating fibers. Macrophages are also seen, many of them with bacilli (arrowhead), but no lymphocytic infiltrate (semithin transverse section stained with toludine blue ×750). B. Electron photomicrograph of the same Figure 4A nerve showing three macrophages (M) carrying large numbers of bacilli and myelin fragments (arrow) lying in separate compartments. No bacilli are seen in the adjacent Schwann cells (S). One of the Schwann cells is seen with a large myelin ring within its cytoplasm (uranyl acetate plus lead citrate stain ×4000).

treated mice (group 3), the lesion obtained was similar to that of TR200 × 5R mice with *M. leprae*. Several macrophages carrying large numbers of *M. leprae* were seen within the endoneurium (Fig. 4A and 4B).

DISCUSSION

This study is a preliminary attempt to establish an experimental leprosy model primarily to get tuberculoid and lepromatous types of granulomatous responses in the nerve. Our results show that it is possible to observe a spectrum of immune responses by immunomodulating the animal in relation to the nerve damage. There was no significant difference observed in the two groups of experiments carried out using human- and armadillo-derived *M. leprae*. The kinetics of the cells migrating into the lesion remain to be explored.

The observation that *M. leprae* were not seen within the Schwann cells even in immunosuppressed mice further raised our curiosity to extend our observations to silica-treated mice in order to immobilize the macrophages. However, our results in this experimental group proved more interesting.

In Swiss white mice on silica treatment following a crush injury there was more or less total retardation of the regenerative activity of the nerve fibers at 2 weeks. This was unlike what has been reported by Muller and Minwegan ([14]) in a rat model. At 4–5 weeks these authors reported a macrophage infiltrate plus nerve regeneration but poor myelin clearance. This difference may be due to the dose of silica used, the time span of study, or the animal species itself.

In our experiment, the addition of *M. leprae* seemed to negate the silica effect, to in-
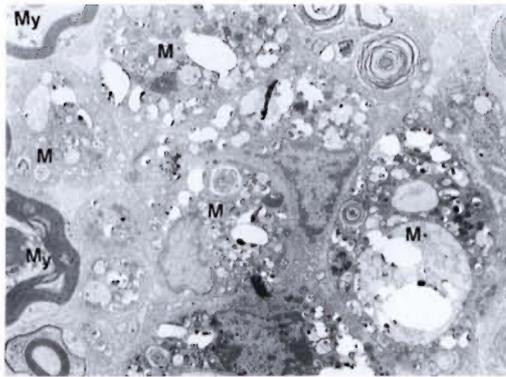
FIG. 5. Electron photomicrograph of part of the sciatic nerve injected intraneurally with two doses of *M. leprae* (10–20 × 10⁶ and 30 × 10⁶) at a 2-week interval. This mouse was immunosuppressed (T200 × 5R) as well as silica treated. Biopsy was obtained 5 weeks after first dose of *M. leprae*. Note the presence of bacilli-laden macrophages (M) showing foamy cytoplasmic changes. Myelinated nerve fibers (My) are seen on one side. This lesion is comparable to a lepromatous-type nerve lesion (uranyl acetate plus lead citrate stain ×4000).

vite a (sub)population of macrophages into the lesions, and to activate the normal course of de-/regenerative activity of the nerve. What was intriguing was the observation of the lack of lymphocytes and epithelioid type of response in these lesions. This observation suggests a role for a specific group of (silica sensitive ?) macrophages in bringing about a tuberculoid type of granulomatous response.

We failed to see *M. leprae* in the morphologically identifiable Schwann cells even in silica-treated mice. It is difficult to explain why *in situ* Schwann cells did not take up any bacteria even though they were viable. In one of our earlier studies, a piece of highly positive lepromatous nerve was grafted onto the sciatic nerve of an immunosuppressed mouse. Although there was good axon innervation into the grafted human nerve, no bacilli were seen in the host Schwann cells even after 6 months of grafting ([17]). However, in the *in vitro* system, both in organized dorsal root ganglion cultures and in monolayer cultures of Schwann cells of mice and humans ([12, 16]), Schwann cells do phagocytose *M. leprae*. In a renewed effort, we injected a second higher dose of *M. leprae* (30 × 10⁶) into the sciatic nerve

of one of the immunosuppressed, silica-treated mice and studied the nerve 5 weeks after the first dose of *M. leprae*. Even in this nerve, although there were carpets of macrophages with *M. leprae* (Fig. 5) lying next to denervated Schwann cells, none were located within the Schwann cells. This is contrary to what we see in human leprous nerves.

In a study of the cell population in Wallerian degeneration, Gibson ([5]) found no evidence for the once quite widely held view that Schwann cells transform into macrophages. Hence, it is unlikely that the macrophages seen in these nerves were transformed Schwann cells. To find the answer to what prevents *M. leprae* from entering the Schwann cells of mice *in situ* would be an interesting area for further study.

SUMMARY

Intraneural injection of 10–20 × 10⁶ viable *Mycobacterium leprae* into the sciatic nerve of normal, unsensitized, Swiss white mice gives rise to a tuberculoid type of granulomatous response in 2 weeks. The same dose of viable *M. leprae* when injected into the sciatic nerves of unsensitized immunosuppressed mice (T200 × 5R) elicited a macrophage response. When macrophages were systemically immobilized using an intraperitoneal injection of silica quartz dust in normal mice, the lesion produced was of the lepromatous type, suggesting a role for the macrophage in the induction of the tuberculoid type of granulomatous response. In all of these *in situ* experiments, *M. leprae* failed to enter the Schwann cells.

RESUMEN

La inyección intraneural de 10–20 millones de bacilos de la lepra viables en el nervio sciático de ratones Swiss-white normales, origina en dos semanas una respuesta de tipo granulomatosa. La misma dosis de *Mycobacterium leprae* viables inyectada en los nervios sciáticos de ratones inmunosuprimidos (T200 × 5R) indujo un respuesta macrofágica. Cuando los macrófagos se inmobilizaron sistémicamente inyectando intraperitonealmente polvo de silicato de cuarzo en ratones normales, la lesión producida fue del tipo lepromatoso sugiriendo un papel de los macrófagos en la inducción de la respuesta granulomatosa de tipo tuberculoide. En todos estos experimentos *in situ* nunca se observó que el *M. leprae* penetrara en las células de Schwann.

RÉSUMÉ

L'injection intra-neurale de 10–20 × 10⁶ organismes de *Mycobacterium leprae* viables dans le nerf sciatique de souris blanches Swiss normales et non sensibilisées donne lieu dans les deux semaines à une réponse granulomateuse de type tuberculoïde. La même dose de *M. leprae* viables injectée dans le nerf sciatique de souris non sensibilisées mais ayant subi une immunosuppression (T200 × 5R) a produit une réponse macrophagique. Quand les macrophages ont été systématiquement immobilisés par l'injection intra-péritonéale de poussière de quartz de silice chez la souris normale, la lésion produite était du type lépromateux, ce qui suggère que le macrophage a un rôle dans la production de la réponse granulomateuse de type tuberculoïde. Dans aucune de ces expérimentations *in situ, M. leprae* n'est entré dans les cellules de Schwann.

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