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Superiority of the Neonatally Thymectomized Lewis Rat (NTLR) to Monitor a Clinical Trial in Lepromatous Leprosy of the Two Regimens of Rifampin and Dapsone[1,2]

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The search for an effective chemotherapeutic regimen capable of successfully eradicating viable *Mycobacterium leprae* from the tissues of lepromatous leprosy patients is hampered by the inability to grow the organism *in vitro*. Furthermore, the testing of new regimens in clinical trials is restricted by the lack of a highly susceptible experimental animal capable of detecting small numbers of viable *M. leprae* in the presence of large numbers of dead *M. leprae*, as often occurs in patient tissues following initial chemotherapy.

Although the immunologically normal mouse has been of great value in many aspects of leprosy research, including the development of new chemotherapeutic drugs for the treatment of human leprosy ([18]), it has only limited value for detecting the presence of low numbers of viable *M. leprae* in tissues from patients with lepromatous leprosy who are undergoing initial chemotherapy. The major limitation of the normal mouse is its inability to detect the growth of *M. leprae* upon administration of 10^6 or more bacilli per foot pad ([7, 16, 22, 23]). This restriction of *M. leprae* multiplication in the mouse foot pad beyond a ceiling of approximately 10^6 organisms appears to be the result of cell-mediated immune (CMI) mechanisms of the host ([11, 13, 20]). This limitation on the number of *M. leprae* that can be inoculated into the mouse foot pad and still allow detection of viable bacilli restricts its use to monitoring specimens from patients in which the proportion of live organisms is 0.1% or greater ([7]). However, when the proportion of viable *M. leprae* is less than 0.1%, the sensitivity of the mouse foot pad assay is greatly diminished. Thus, early in therapy skin biopsies from patients still harboring significant numbers (10^8 to 10^9) of viable *M. leprae* within their tissues may not demonstrate viable *M. leprae* following passage into mouse foot pads. However, later, as a result of selective clearance of dead organisms, the proportion of viable bacilli within patient tissues increases to 0.1% of the total bacilli, and viable bacilli can again be detected by mouse inoculation ([14]).

In a previous study, Fieldsteel and McIntosh ([9]) showed that the neonatally thymectomized Lewis rat (NTLR) exhibited an increased susceptibility to *M. leprae* infection as compared with the normal mouse. It was demonstrated that the NTLR will regularly show growth from as few as five viable *M. leprae* injected together with 10^7 heat-killed *M. leprae* ([4, 7]). These studies suggested that the NTLR and the more-recently described congenitally athymic rat ([2, 3]) might be more sensitive monitors of initial chemotherapy of lepromatous patients than the routine mouse assay. The present study was performed to test this hypothesis by comparing the NTLR, the congenitally athymic rat, and the normal mouse for their ability to detect low percentages of viable *M. leprae* in tissues of lepromatous patients early in a clinical trial using two potentially bactericidal regimens consisting of rifampin plus dapsone.

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[2] These data appeared in preliminary and summary form as "A comparative study of four rodent systems to monitor initial therapy of lepromatous leprosy: in search of a more sensitive system to assess bacterial viability." Acta Leprol. (Genève) **2** (1984) 319–325.

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MATERIALS AND METHODS

Study population. Fifteen untreated patients classified according to the dermal histopathology criteria of Ridley and Jopling ([21]) as either polar lepromatous (LLp) or subpolar lepromatous (LLs) were entered in the trial. The patients were randomly placed into two treatment groups. One group (seven patients) received a single initial dose of 1500 mg rifampin plus 100 mg dapsone daily (Treatment A). The other group (eight patients) received 900 mg rifampin weekly plus daily doses of 100 mg dapsone (Treatment B). Skin biopsies (>10 mm diameter) were taken from the most active skin lesions before and at various times after initiation of chemotherapy (generally at 3 or 4 days, 1 week, 2 weeks, and 4 weeks).

Animals. Pregnant inbred rats of the Wistar/Lewis strain were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts, U.S.A. These animals were specific pathogen-free with a defined microflora. BALB/c mice were obtained from our own colonies. The Lewis rats were thymectomized before the animals were 24 hr old—in most instances between 5 and 16 hr after birth as described previously ([6]).

Athymic rats were obtained from our own breeding colony that was originally established from a small breeding nucleus of rats heterozygous for the *rnu* gene, obtained from the Laboratory Animal Centre, Carshalton, England. The animals used for this study were of mixed genetic backgrounds ([3]) and were housed under specific pathogen-free conditions in laminar flow isolators (Lab Products Inc., Rochelle Park, New Jersey, U.S.A.).

***M. leprae* assay procedures.** The methods of tissue processing, inoculation, and counting of the *M. leprae* were those described by Shepard ([22]) and Shepard and McRae ([29]). On the day of biopsy, the number of acid-fast bacilli (AFB) per patient biopsy was determined, and various concentrations of *M. leprae* were inoculated into BALB/c mice, NTLR, and athymic rats.

In the standard mouse assay, five mice were inoculated with 5×10^3 AFB/foot pad (FP) in both hind feet (BHF). The foot pads from two mice were harvested at approximately 12 months, and the number of AFB/FP determined. If this showed an increase of greater than fivefold over the number inoculated, then the original inoculum was reported as positive for viable *M. leprae.*

In assays using the NTLR or athymic rat, three rats per dose of *M. leprae* were inoculated in BHF. The number of *M. leprae* inoculated into immunosuppressed animals ranged from 10^5 to 10^7 AFB/FP, depending on the number of AFB/patient biopsy. In most cases, the largest possible inoculum was inoculated into BHF of three rats; when availability of rats and inoculum permitted, tenfold fewer *M. leprae* were inoculated into three additional rats. Both hind feet from a single rat from each inoculum were harvested at one year, and bacilli in each FP were counted individually; subsequent rat foot pads were harvested and AFB determined upon demise of the remaining rats. The inoculum was reported as positive for viable *M. leprae* when the increase over the original inoculum was greater than fourfold.

In a previous study employing NTLR to determine the presence of viable *M. leprae* in animals receiving drug therapy ([8]), it became apparent that the criteria for multiplication of *M. leprae* in intact animals could not be utilized to assess multiplication in NTLR. If we inoculated NTLR with up to 5×10^7 *M. leprae* from NTLR receiving various chemotherapeutic regimens, at harvest a year or more after inoculation we frequently recovered fewer organisms than the number originally inoculated. However, when 5×10^3 of the recovered organisms were subpassaged to foot pads of normal mice, unequivocal growth was often observed. Thus, in those cases where the original passage into NTLR (or athymic rats) failed to show unequivocal multiplication (less than a fourfold increase over the original inoculum and greater than 10^5 *M. leprae* detected), we attempted to ascertain the presence of viable *M. leprae* by an additional subpassage of the recovered organisms from primary rat passage into mice as described above. If subsequent mouse foot pad harvests showed greater than fivefold increases, then the original inoculum (obtained from patient material) was considered positive for viable *M. leprae.*

TABLE 1. *Results of direct passage and subpassage of patient biopsies into mice, NTLR, and nude rats from patients on Treatment A.*[a]

Patient no.	Days after initiation of therapy	Total AFB per biopsy[b]	Routine mouse assay[c]	No. of animals demonstrating multiplication over total number of animals injected with various concentrations of *M. leprae*						Viable bacilli detected[e]
				NTLR[d]			Nude[d]			
				10^5	10^6	10^7	10^5	10^6	10^7	
1	0	6.9×10^6	Pos.	—[f]	—	—	—	—	—	Yes
	3	1.2×10^8	Neg.	0/3	0/3	—	—	—	—	No
	15	4.0×10^5	Neg.	0/3	—	—	—	—	—	No
	29	1.0×10^7	Neg.	0/3	—	—	—	—	—	No
2	0	3.0×10^8	Pos.	—	—	—	—	—	—	Yes
	3	9.3×10^7	Neg.	—	0/2	—	—	—	—	No
	7	1.6×10^8	Neg.	1/3 (1/2)	0/3 (1/1)*[g]	—	—	—	—	Yes
	14	9.6×10^7	Neg.	0/3	0/2 (0/2)	—	—	—	—	No
	28	1.1×10^8	Neg.	0/3 (0/1)	0/3 (0/1)	—	—	—	—	No
3	0	1.8×10^7	Pos.	—	—	—	—	—	—	Yes
	2	1.3×10^8	Neg.	0/3	0/3 (0/2)	—	0/1	0/2	—	No
	7	1.6×10^8	Neg.	—	0/3	0/3 (0/1)	0/2	0/2	—	No
	14	7.1×10^7	Neg.	0/2	0/3 (0/1)	—	0/2	0/2	—	No
	28	7.6×10^7	Neg.	0/3 (0/1)	0/3 (0/1)	—	0/2	0/1	—	No
4	0	3.2×10^7	Pos.	—	—	—	—	—	—	Yes
	3	5.5×10^7	Neg.	0/3	0/3 (0/2)	—	—	0/3	—	No
	7	4.7×10^6	Neg.	0/3 (0/1)	—	—	0/3	—	—	No
	14	3.0×10^7	Neg.	0/3	0/3 (0/1)	—	0/2	—	—	No
	28	4.7×10^7	Neg.	0/4	0/2	—	0/3	—	—	No
5	0	1.0×10^8	Pos.	3/3	3/3	—	—	—	—	Yes
	3	4.1×10^7	Neg.	1/3 (1/1)	2/3 (3/3)	—	—	—	—	Yes
	7	4.1×10^8	Neg.	—	3/3 (1/1)	0/3 (1/1)*	—	—	—	Yes
	15	3.0×10^8	Neg.	—	1/3 (1/1)	0/3 (0/3)	—	—	—	Yes
	28	4.1×10^8	Neg.	—	1/3 (2/3)	0/3 (2/2)	—	—	—	Yes
6	0	2.4×10^7	Pos.	—	—	—	—	—	—	Yes
	8	5.2×10^7	Neg.	0/3	0/3 (0/1)	—	—	—	—	No
	16	7.7×10^7	Neg.	0/3	0/3 (0/1)	—	—	—	—	No
	28	3.2×10^7	Neg.	0/3	0/3	—	—	—	—	No
7	0	3.2×10^8	Pos.	—	—	—	—	—	—	Yes
	6	4.8×10^8	Neg.	1/3 (0/1)	0/3 (3/3)*	—	0/2 (0/1)	0/3 (0/1)	—	Yes
	9	2.9×10^8	Neg.	0/3	1/3 (1/1)	—	0/2	0/2 (0/1)	—	Yes
	16	3.2×10^8	Neg.	0/3 (0/1)	0/3 (2/2)*	—	0/3 (0/1)	0/2 (0/2)	—	Yes
	30	2.4×10^8	Neg.	0/3 (0/2)	0/3 (1/3)*	—	0/3	0/2 (0/1)	—	Yes

[a] Patients received a single initial dose 1500 mg rifampin plus daily doses of 100 mg dapsone.

[b] Punch biopsies (10 mm diameter) were homogenized and the total number of AFB per specimen was determined.

[c] Five BALB/c mice were inoculated in each hind foot pad with 5×10^3 AFB from the patient biopsy. Two mice were harvested at 6 months. If the number of AFB showed an increase of greater than fivefold, then the inoculum was reported as positive for viable *M. leprae.* If the harvest showed less than a fivefold increase, the remaining 3 mice were harvested at one year. If this harvest showed less than a fivefold increase, the inoculum was reported as negative for viable *M. leprae.*

[d] In most cases, 3 NTLR or nude rats per *M. leprae* concentration were inoculated with 10^5 to 10^7 AFB in BHF. At 12, 18, and 24 months, at least one rat from each concentration was harvested and BHF were examined for AFB. The inoculum was reported as positive for viable *M. leprae* when an increase greater than fourfold was observed.

[e] Indicates whether biopsies were positive or negative for viable *M. leprae* by any of the assay methods used.

[f] Denotes value not determined.

[g] In those instances were results from the primary passage into rats showed less than a fourfold increase, an additional subpassage of rat-derived material was made into normal BALB/c mice using the routine mouse assay. The results of this subpassage are shown in parentheses. An asterisk (*) denotes those cases in which viable *M. leprae* were detected by subpassage of rat FP material into normal mice although direct passage of patient biopsy material into NTLR (or nude rat) foot pads proved negative.

RESULTS

The results obtained from the 15 patients following inoculation of biopsy specimens into mice, NTLR, and congenitally athymic (nude) rats are shown in Tables 1 and 2. Table 1 shows the data from the group of patients receiving Treatment A, and Table 2 shows the data from the group of patients receiving Treatment B.

TABLE 2. *Results of direct passage and subpassage of patient biopsies into mice, NTLR, and nude rats from patients on Treatment B.*[a]

Patient no.	Days after initiation of therapy	Total AFB per biopsy[b]	Routine mouse assay[c]	No. of animals demonstrating multiplication over total number of animals injected						Viable bacilli detected[c]
				NTLR[d]			Nude[d]			
				10^5	10^6	10^7	10^5	10^6	10^7	
8	0	8.0×10^7	Pos.	3/5 (1/1)	3/3	—[f]	—	—	—	Yes
	3	1.4×10^8	Pos.	0/6 (1/1)*[g]	0/3 (0/1)	—	—	—	—	Yes
	8	1.2×10^8	Neg.	0/3	0/3	—	—	—	—	No
	13	1.8×10^8	Neg.	0/3	0/3 (0/1)	—	—	—	—	No
	29	1.0×10^8	Neg.	0/3	0/2 (0/1)	—	—	—	—	No
9	0	2.7×10^8	Pos.	—	—	—	—	—	—	Yes
	4	2.8×10^8	Neg.	—	2/3 (2/2)	0/3 (2/2)	—	—	—	Yes
	7	9.8×10^8	Neg.	—	1/3 (3/3)	0/3 (0/2)	—	—	—	Yes
	14	1.6×10^8	Neg.	—	1/3 (3/3)	1/3 (1/3)	—	—	—	Yes
	28	2.7×10^8	Neg.	—	0/3 (0/1)	0/3 (2/3)*	—	—	—	Yes
10	0	5.1×10^6	Pos.	—	—	—	—	—	—	Yes
	4	2.6×10^7	Neg.	—	3/6 (2/3)	—	—	—	—	Yes
	7	3.2×10^7	Neg.	—	2/3 (1/1)	1/2 (1/1)	—	—	—	Yes
	14	1.7×10^7	Neg.	0/3	0/3	—	—	—	—	No
	28	9.2×10^6	Neg.	0/6	—	—	—	—	—	No
11	0	8.4×10^8	Pos.	—	—	—	—	—	—	Yes
	2	6.9×10^8	Neg.	—	1/3 (2/3)	1/3 (3/3)	—	0/3	0/3 (0/1)	Yes
	7	6.3×10^8	Neg.	—	1/3 (1/2)	0/3 (2/2)*	—	0/2	0/2 (0/1)	Yes
	14	7.3×10^8	Neg.	—	0/3 (1/1)*	1/6 (3/5)	—	0/4	—	Yes
	28	ND	Neg.	—	0/3 (1/3)*	0/3 (0/3)	—	0/2	0/2	Yes
12	0	6.6×10^7	Pos.	—	—	—	—	—	—	Yes
	3	2.0×10^8	Neg.	—	0/4	—	0/3	0/3	—	No
	7	1.8×10^8	Neg.	0/3 (0/1)	1/3 (1/3)	—	0/2	0/2 (0/1)	—	Yes
	14	1.9×10^8	Neg.	0/3 (0/1)	0/3 (0/1)	—	—	—	—	No
	28	1.8×10^8	Neg.	0/3	0/3	—	0/2	0/2 (0/1)	—	No
13	0	2.2×10^7	Pos.	—	—	—	—	—	—	Yes
	5	3.6×10^8	Neg.	—	2/3 (2/2)	0/3 (1/2)*	—	—	—	Yes
	7	3.6×10^8	Neg.	—	1/3 (1/1)	0/3 (2/3)*	—	—	—	Yes
	14	2.1×10^8	Neg.	—	1/3	0/3 (3/3)*	—	—	—	Yes
	28	3.5×10^8	Neg.	—	0/2	0/3 (1/2)*	—	—	—	Yes
14	0	3.4×10^8	Pos.	—	—	—	—	—	—	Yes
	6	4.4×10^7	Neg.	0/3	0/3 (2/2)*	—	0/3 (0/1)	0/3 (1/2)*	—	Yes
	9	2.8×10^7	Neg.	0/3	—	—	0/1	—	—	No
	16	7.9×10^7	Neg.	0/3 (0/1)	0/3 (0/1)	—	0/3	0/3	—	No
	30	2.5×10^8	Neg.	0/3	0/3 (0/2)	—	0/1	0/3 (0/2)	—	No
15	0	3.3×10^7	Pos.	—	—	—	—	—	—	Yes
	3	6.0×10^7	—	1/2 (1/1)	2/2	—	2/3	0/3 (1/1)	—	Yes
	7	1.3×10^8	Pos.	2/3 (1/2)	1/2 (1/1)	—	2/3	0/2 (0/1)	—	Yes
	14	5.6×10^7	Neg.	0/3 (1/2)*	1/3	—	0/3 (1/1)*	0/3	—	Yes
	29	7.4×10^7	Neg.	0/3	0/3 (1/1)*	—	0/3	0/3	—	Yes

[a] Patients received daily doses of 100 mg dapsone plus 900 mg rifampin weekly.

[b] Punch biopsies (10 mm diameter) were homogenized and the total number of AFB per specimen was determined.

[c] Five BALB/c mice were inoculated in each hind foot pad with 5×10^3 AFB from the patient biopsy. Two mice were harvested at 6 months. If the number of AFB showed an increase of greater than fivefold, then the inoculum was reported as positive for viable *M. leprae*. If the harvest showed less than a fivefold increase, the remaining 3 mice were harvested at one year. If the harvest showed less than a fivefold increase, the inoculum was reported as negative for viable *M. leprae*.

[d] In most cases, 3 NTLR or nude rats per *M. leprae* concentration were inoculated with 10^5 to 10^7 AFB in BHF. At 12, 18, and 24 months, at least one rat from each concentration was harvested and BHF were examined for AFB. The inoculum was reported as positive for viable *M. leprae* when an increase greater than fourfold was observed.

[e] Indicates whether biopsies were positive or negative for viable *M. leprae* by any of the assay methods used.

[f] Denotes value not determined.

[g] In those instances were results from the primary passage into rats showed less than a fourfold increase, an additional subpassage of rat-derived material was made into normal BALB/c mice using the routine mouse assay. The results of this subpassage are shown in parentheses. An asterisk (*) denotes those cases in which viable *M. leprae* were detected by subpassage of rat FP material into normal mice although direct passage of patient biopsy material into NTLR (or nude rat) foot pads proved negative.

TABLE 3. *Results of assays for viable* M. leprae *on biopsies from patients treated with 100 mg dapsone daily and 1500 mg rifampin on day 0 (Treatment A).*

Patient no.	Day(s) after treatment									
	Day 0		Days 3–6		Days 7–9		Days 14–16		Days 28–30	
	I[a]	II[b]	I	II	I	II	I	II	I	II
1	+[c]	ND[d]	0[e]	0	0	ND	0	0	0	0
2	+	ND	0	0	0	+	0	0	0	0
3	+	ND	0	0	0	0	0	0	0	0
4	+	ND	0	0	0	0	0	0	0	0
5	+	+	0	+	0	+	0	+	0	+
6	+	ND	ND	ND	0	0	0	0	0	0
7	+	ND	0	+	0	+	0	+	0	+
Totals[f]	7/7	1/1	0/6	2/6	0/7	3/6	0/7	2/7	0/7	2/7
Viable *M. leprae* detected[g]	7/7 = 100%		2/6 = 33%		3/6 = 50%		2/7 = 29%		2/7 = 29%	

[a] Assay I = 5×10^3 AFB per foot pad were inoculated into BHF of 5 BALB/c mice. Foot pads from 2 mice were harvested at six months. If AFB counts showed more than a fivefold increase over the inoculum, they were reported as positive. If multiplication of *M. leprae* was less than fivefold, the remaining mice were harvested at one year. If AFB counts still showed less than a fivefold increase, results were then reported as negative (0).

[b] Assay II = 10^5 to 10^7 AFB per foot pad were inoculated into BHF of NTLR or nude rats. Foot pads were harvested at 12, 18, 24 months. If AFB counts showed more than a fourfold increase over inoculum, the biopsy was reported as positive. In some cases, if multiplication of inoculum was less than fourfold, the samples were subpassaged into 5 normal BALB/c mice at 5×10^3 per FP and treated as in Assay I.

[c] Viable *M. leprae* were detected.

[d] Not determined.

[e] Viable *M. leprae* were not detected.

[f] Patients positive for viable *M. leprae* by each assay per number of patients tested.

[g] Patients positive for persisting *M. leprae* by either assay per number of patients tested. Also designated as percent positive.

Detection of viable *M. leprae* in patient biopsy material using mice and NTLR as the *in vivo* assay system. In 100% (15/15) of the patient biopsies collected prior to chemotherapy, viable *M. leprae* were detected in the routine mouse assay. However, the proportion of viable *M. leprae* was drastically reduced after the initiation of chemotherapy because only two biopsies (from patient no. 8 on day 8 and patient no. 15 on day 7) of the 58 tested exhibited viable bacilli following inoculation into mouse foot pads.

In contrast, 22 of 58 biopsies taken after the initiation of therapy from nine patients (nos. 2, 5, 7, 9, 10, 11, 12, 13, and 15) were positive for viable *M. leprae* following direct passage into NTLR at various times after treatment. In eight biopsies (patients nos. 7, 8, 9, 11, 13, 14, and 15), viable *M. leprae* were not detected by direct passage of patient biopsy material into NTLR, but were detected after a further subpassage of NTLR-derived material (AFB harvested from NTLR showing equivocal results) into mice.

Larger inocula were most commonly associated with the demonstration of viable bacilli. Of the 15 instances in which NTLR or their passage mice confirmed viability and both 10^5 and 10^6 bacilli were inoculated, there was only one instance in which the 10^5 inoculum grew but 10^6 did not, six instances in which the 10^6 inoculum grew but 10^5 did not, and eight instances in which both the 10^5 and 10^6 inocula grew. In those instances in which both 10^6 and 10^7 bacilli were used for inoculation and viable *M. leprae* detected (16 biopsies total), there were three instances in which the 10^6 inoculum grew and the 10^7 did not, two instances in which the 10^7 inoculum grew and the 10^6 did not, and eleven instances in which both 10^6 and 10^7 inocula grew. These results suggest that high numbers of AFB from patient biopsy material of 10^6 and 10^7 are equivalent in enabling the detection of viable *M. leprae*; although not statistically significant ($p = 0.1$), lower AFB concentrations (10^5) may be, in this respect, inferior.

Detection of viable *M. leprae* in patient biopsy material using the congenitally

TABLE 4. *Results of assays for viable* M. leprae *on biopsies from patients treated with 100 mg dapsone daily and 900 mg rifampin weekly (Treatment B).*

Patient no.	Day(s) after treatment									
	Day 0		Days 2–6		Days 7–9		Days 13–16		Days 28–30	
	I[a]	II[b]	I	II	I	II	I	II	I	II
8	+[c]	+	+	+	0[d]	0	0	0	0	0
9	+	ND[e]	0	+	0	+	0	+	0	+
10	+	ND	0	+	0	+	0	0	0	0
11	+	ND	0	+	0	+	0	+	0	+
12	+	ND	0	0	0	+	0	0	0	0
13	+	ND	0	+	0	+	0	+	0	+
14	+	ND	0	+	0	0	0	0	0	0
15	+	ND	ND	+	+	+	0	+	0	+
Totals[f]	8/8	1/1	1/7	7/8	1/8	6/8	0/8	4/8	0/8	4/8
Viable *M. leprae* detected[g]	8/8 = 100%		7/8 = 88%		6/8 = 75%		4/8 = 50%		4/8 = 50%	

[a] Assay I = 5×10^3 AFB per foot pad were inoculated into BHF of 5 BALB/c mice. If subsequent AFB counts showed more than a fivefold increase over the inoculum, they were reported as positive. If multiplication of *M. leprae* was less than fivefold, results were then reported as negative (0).

[b] Assay II = 10^5 to 10^7 AFB per foot pad were inoculated into BHF of NTLR or nude rats. If subsequent AFB counts showed more than a fourfold increase over inoculum, the biopsy was reported as positive. In some cases, if multiplication of inoculum was less than fourfold and greater than 10^5 *M. leprae* found, the samples were subpassaged into 5 normal BALB/c mice at 5×10^3 per FP and treated as in Assay I.

[c] Viable *M. leprae* were detected.

[d] Viable *M. leprae* were not detected.

[e] Not determined.

[f] Patients positive for viable *M. leprae* by each assay per number of patients tested.

[g] Patients positive for persisting *M. leprae* by either assay per number of patients tested. Also designated as percent positive.

athymic (nude) rat as the *in vivo* assay system. In this study, depending on the availability of athymic animals, we were able to compare the sensitivity of the NTLR and the nude rat using biopsies from seven patients (nos. 3, 4, 7, 11, 12, 14, and 15). The results show that the athymic rat lacks the sensitivity to serve as a monitor for detecting persisting *M. leprae* in the tissues of patients undergoing chemotherapy. Of the total of 27 biopsies passed into nude rats, only two (from patient no. 15) showed more than a fourfold increase over the original inoculum after direct passage, as compared to nine in NTLR ($p < 0.02$, McNemar test[15]). In the case of the biopsy obtained on day 7 from this patient, the mouse assay also detected viable *M. leprae.* Subpassage of foot pad material from nude rats showing equivocal growth (less than a fourfold increase over the original inoculum) to mice had little effect on the sensitivity of the nude rat in that only two additional biopsies proved positive for viable *M. leprae* (patient no. 14 on day 6 and patient no. 15 on day 14). Thus, *in toto,* the nude rat detected less (4/27) biopsies with viable bacilli than the NTLR (14/27) ($p = 0.002$, McNemar test[15]). The nude rat never detected a positive specimen that was negative for the NTLR when both types of animals were run in parallel. However, there were a number of instances in which the NTLR detected the presence of viable *M. leprae* and the nude rat did not.

Efficacy of the two treatment regimens for eradication of persisting *M. leprae.* Tables 3 and 4 compare the two methods of chemotherapy used for treatment of lepromatous leprosy in this study. The tables show the percentage of patients with viable *M. leprae* at various times after initiation of chemotherapy, as determined by either the mouse assay (Assay I) or by passage into NTLR and nude rats (Assay II). Although the results suggest that Treatment A (Table 3), which consisted of a single high dose of rifampin plus daily dapsone, may be more effective in eradicating *M. leprae* than Treatment B (Table 4), which consisted of intermittent administration of lower doses of rifampin in combination with daily dap-

sone, the difference is not significant due to the small sizes of the treatment groups. However, the percentage of patients exhibiting biopsies positive for viable *M. leprae* was lower for the patients receiving Treatment A at all four time periods assayed. Viable bacilli were detected by NTLR with subsequent mouse passage in 9 of 26 (35%) skin biopsies following the single-dose rifampin regimen (Treatment A) and in 21 of 32 (66%) skin biopsies following the multiple-dose rifampin regimen (Treatment B).

The tables further illustrate the increased sensitivity of immunosuppressed NTLR (Assay II) over the normal mouse (Assay I) for determining low numbers of viable *M. leprae* in the presence of killed *M. leprae* as observed in patient tissues following initial chemotherapy. Table 3 shows that the mouse assay uniformly failed to detect viable *M. leprae* after initiation of chemotherapy; whereas the positive biopsies for the NTLR assay ranged from 29% to 50% for all time periods tested. Similarly, Table 4 indicates that the sensitivity of the mouse assay was much less than that of the NTLR assay, which showed levels of *M. leprae* detection ranging from 50% to 88% of the biopsies tested compared with 0% to 14% for the mouse assay.

During the late stages of this study, we also investigated whether the intact mouse might be substituted for the NTLR as a means of detecting small numbers of viable bacilli in patient tissues (data not shown). These preliminary experiments were based on an observation of Fieldsteel and Colston ([5]) that low numbers of viable *M. leprae* (10 to 100) could be detected even in the presence of high numbers of heat-killed organisms (10^5 to 10^7) if the mouse foot pad assay was followed by an additional subpassage of mouse foot pad-derived material into mice. This method of assaying for viable *M. leprae* was applied in this study. Using a limited number of biopsy specimens, we observed an increase in detection over that found in the standard mouse foot pad assay. Fourteen biopsies were inoculated in parallel into mice utilizing large inocula (10^4 to 10^6) and NTLR utilizing large inocula (10^5 to 10^7). Five biopsies demonstrated viable bacilli in mice receiving large inocula following subsequent mouse passage. The NTLR demonstrated viable *M. leprae* in the same five instances and in an additional six instances. Substantiation of *M. leprae* viability by NTLR was determined directly in seven instances but required mouse passage in four instances.

DISCUSSION

Since Shepard's description of the experimental infection that results after *M. leprae* inoculation into the mouse foot pad ([22]), a number of clinical trials have been performed using the mouse as a means of monitoring *M. leprae* viability within the tissues of patients undergoing short-term chemotherapy for lepromatous leprosy ([24–26, 30]). In these studies, the loss of viability of organisms obtained from serial skin biopsies of patients receiving treatment with either dapsone, the intramuscular repository diacetyl-derivative of dapsone (DADDS), or clofazimine was shown to occur over a period of a few months as determined by passage into mouse foot pads. In contrast, rifampin was found highly bactericial for *M. leprae* since bacilli obtained from patient biopsy specimens two to three days after initiation of treatment with daily or single large doses of rifampin often failed to multiply following inoculation into mouse foot pads ([19, 27, 28]). Although the above studies suggest that certain chemotherapy regimens are capable of rapidly eliminating viable *M. leprae* from the tissues of lepromatous leprosy patients, other more long-term observations of chemotherapy efficacy have shown that this is not the case whether ten years of daily dapsone therapy ([33]) or two or more years of daily rifampin ([34]) are employed. These studies were in part monitored by the thymectomized x-irradiated, bone-marrow reconstituted mouse as described by Rees ([17]). Although the mechanisms responsible for these "persisters" have yet to be elucidated ([31]), it is thought that such "persisters" may be capable of inducing clinical relapse if therapy is ever discontinued, and hence life-long treatment for lepromatous leprosy is advised by most leprologists. Therefore, the development of a sensitive monitor for persisting *M. leprae* in patient tissues would be of value for evaluating the efficacy of current chemotherapeutic regimens and the development of new

antileprosy drugs for the treatment of lepromatous leprosy.

The objective of this study was to compare the sensitivity of two alternative animal assay systems with that of the commonly employed mouse foot pad method. The results show that the NTLR is a more sensitive monitor than the normal mouse for detecting low numbers of viable *M. leprae* in tissues from leprosy patients undergoing short-course chemotherapy. The direct inoculation of NTLR foot pads with patient-derived material detected viable *M. leprae* in 38% (22/58) of the biopsies tested, as compared with 4% (2/57) upon direct inoculation into mouse foot pads. Furthermore, the sensitivity of detection utilizing the NTLR was substantially increased by including an additional subpassage of inocula containing AFB from the NTLR to the mouse. This was shown by the increased detection of positive specimens to 52% (30/58) of the total tested. However, there was one instance (patient no. 8 on day 3) in which direct inoculation into mouse foot pads detected viable *M. leprae* but direct inoculation into NTLR foot pads did not. This was the only case in which the mouse proved more sensitive than the NTLR. Thus, even though NTLR may exhibit a range of susceptibility to *M. leprae* infection, the NTLR is still superior to the normal mouse as a monitor of persisting *M. leprae.*

In a previous publication by Fieldsteel, *et al.* ([10]) it was demonstrated that neonatal thymectomy of Lewis rats results in a severe depletion of both circulating white blood cells (WBC) and thymus-derived lymphocytes. However, it was also shown that the degree of immunosuppression and susceptibility to *M. leprae* infection varied considerably among Lewis rats that had undergone thymectomy. Therefore, during the last few years we have been investigating whether or not the congenitally athymic rat could serve as a possible animal model for multibacillary leprosy. Previous studies by ourselves and others ([1, 12, 32]) have shown that the athymic rat exhibits certain properties normally associated with an immunologic deficiency. In a recent publication ([2]) it was also observed that the athymic rat is similar to the NTLR in that it exhibits a disseminated infection following intravenous inoculation with viable *M. leprae.* On the basis of these observations, the athymic rat was included in this study to determine its sensitivity for detecting low numbers of viable *M. leprae* in the presence of high numbers of killed bacilli.

However, replacement of the NTLR with the congenitally athymic rat using the same method of assay proved unsuccessful. The reason for this lack of sensitivity exhibited by the athymic rat is unknown. However, the athymic rat has been shown to exhibit a high level of innate resistance to xenogenic tumor cells derived from human tumor cell lines ([1]) and has also demonstrated a capacity to limit multiplication when the numbers of mycobacteria reach levels of approximately 10^8 per site ([2]). Whether this mechanism involves elevated levels of macrophage activity or maturation of T-cell precursors in the absence of intrathymic maturation has yet to be elucidated.

Although not statistically significant, the regimen consisting of a single dose of rifampin plus daily dapsone (Regimen A) resulted in a lower percentage of biopsies found to contain viable *M. leprae* at each of the four sampling intervals. All 8 of the subjects receiving Regimen B were found to harbor viable *M. leprae* at least once after the initiation of therapy, while only 3 of 7 patients treated with Regimen A exhibited viable bacilli after therapy commenced. Future studies should utilize the NTLR in a similar manner as described here to monitor initial chemotherapy trials with regimens that might prove more potent than those employed in this study. Indeed, regimens that eliminate viable *M. leprae* more regularly and rapidly from skin biopsies inoculated into the NTLR might prove efficacious in preventing persisters and permit safe discontinuation of therapy.

In conclusion, this report has shown that the NTLR is a more sensitive monitor than the mouse foot pad assay for detecting low fractions of persisting *M. leprae* within tissues of lepromatous patients undergoing initial chemotherapy, and that its increased sensitivity may provide a more accurate indication of the efficacy of antileprosy regimens.

SUMMARY

The ability of the neonatally thymectomized Lewis rat (NTLR) and the congenitally athymic (nude) rat systems to detect low numbers of viable *Mycobacterium leprae* in tissues from lepromatous leprosy patients undergoing short-course chemotherapy was compared with that of the commonly employed mouse foot pad assay. Fifteen previously untreated lepromatous patients were randomly assigned to treatment regimens of either a single initial 1500 mg dose of rifampin plus daily doses of 100 mg of dapsone, or weekly doses of 900 mg of rifampin plus daily doses of 100 mg of dapsone. Four skin biopsies from each patient taken sequentially up to one month after initiation of therapy were used as the source of the *M. leprae* inocula. Only 2 of 57 skin biopsies (2%) proved positive for viable *M. leprae* following direct inoculation into mouse foot pads. However, 30 of 58 patient biopsies (52%) provided positive for viable *M. leprae* following direct passage into NTLR foot pads or in subsequent mouse subpassage. In contrast, the nude rat was observed to be a poor monitor of such trials. Although not statistically significant, the regimen consisting of a single dose of rifampin plus daily dapsone resulted in a lower percentage of biopsies found to contain viable *M. leprae* at each of the four sampling intervals.

RESUMEN

Se comparó la eficiencia del sistema de las ratas Lewis timectomizadas al nacimiento (RLTN) y la del sistema de las ratas congénitamente atímicas (desnudas), con la eficiencia del método de inoculación en la almohadilla plantar del ratón para detectar números bajos de *Mycobacterium leprae* en los tejidos de pacientes lepromatosos sujetos a esquemas cortos de tratamiento. Quince pacientes sin tratamiento previo se asignaron al azar a un esquema de tratamiento con una sola dosis inicial de 1500 mg de rifampina más dosis diarias de 100 mg de dapsona, o a un esquema de tratamiento con 900 mg de rifampina más dosis diarias de 100 mg de dapsona. Como fuente de inóculo de *M. leprae* se usaron 4 biopsias de piel, tomadas una por semana hasta cubrir un mes después de iniciada la terapia. Sólo 2 de 57 biopsias (2%) tuvieron bacilos viables cuando se usó el método de la almohadilla plantar del ratón, sin embargo, 30 de 58 biopsias (52%) tuvieron bacilos viables cuando se inocularon en las almohadillas plantares de las RLTN y en los pasos subsecuentes en las almohadillas plantares del ratón. En contraste, las ratas desnudas no resultaron adecuadas para los fines del estudio. El esquema consistente en una sóla dosis de rifampina más dosis diarias de dapsona condujo a una menor frecuencia (pero sin significancia estadística) de biopsias con *M. leprae* viables en cada intervalo de muestreo.

RÉSUMÉ

On a comparé dans quelle mesure l'inoculation au rat Lewis nouveau-né thymectomisé (NTLR) et l'inoculation au rat glabre congénitalement athymique, permettaient de mettre en évidence un petit nombre de *Mycobacterium leprae* viables dans des tissus récoltés chez des malades atteints de lèpre lépromateuse soumis à une chimiothérapie de courte durée, par rapport à l'épreuve d'inoculation au coussinet plantaire de la souris communément utilisée. Quinze malades lépromateux, non traités au préalable, ont été attribués au hasard à un traitement consistant soit d'une dose initiale de 1500 mg de rifampine suivie de doses quotidiennes de 100 mg de dapsone, soit à des doses hebdomadaires de 900 mg de rifampine accompagnées de doses quotidiennes de 100 mg de dapsone. Quatre biopsies cutanées ont été prélevées chez chaque malade, successivement, jusqu'à un mois après le début de la thérapeutique. Ces biopsies ont servi comme source des inoculats de *M. leprae.* A la suite de l'inoculation directe dans le coussinet plantaire de la souris, deux biopsies cutanées seulement sur 57 (2%), se sont révélées positives pour des *M. leprae* viables. Par contre, 30 parmi 58 biopsies (52%) ont permis de mettre en évidence des *M. leprae* viables à la suite du passage direct dans les coussinets plantaires des rats de Lewis (NTLR) ou dans des passages secondaires ultérieurs chez la souris. Le rat glabre est apparu comme une système peu intéressant pour de telles épreuves. Les régimes thérapeutiques consistant en une dose unique de rifampine suivie de dapsone quotidienne ont entraîné une proportion plus faible de biopsies positives contenant *M. leprae* viable, et ceci à chacun des intervalles successifs d'échantillonnage; ces derniers résultats ne sont cependant pas significatifs.

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