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Detection of Persisting *Mycobacterium leprae* by Inoculation of the Neonatally Thymectomized Rat

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The neonatally thymectomized rat (NTR) was shown by Fieldsteel and his colleagues ([2, 3]) to be more susceptible than is the immunologically normal mouse to infection by *Mycobacterium leprae,* in terms of its ability to permit multiplication of the organisms from inocula $>10^4$ organisms per foot pad. If the susceptibility of the NTR to infection by *M. leprae* were greater than that of the thymectomized-irradiated and bone-marrow-reconstituted (T900R) mouse—i.e., if *M. leprae* multiplied more consistently in the foot pad of the NTR inoculated with $\geq 10^5$ organisms—it might then be possible to detect persisting *M. leprae* in the tissues of patients with lepromatous leprosy during effective antimicrobial treatment with a greater frequency than is presently possible by inoculation of T900R mice.

A small trial of chemotherapy was carried out among patients with untreated lepromatous leprosy in San Francisco, in the course of which lesions were biopsied, and organisms recovered from the biopsy specimens were inoculated into the foot pads of both normal mice and NTR. The results of the trial have been presented elsewhere ([5, 6]). The purpose of this paper is to present an additional analysis of those results, which permits both an indirect comparison of the sensitivity of the NTR with that of the T900R mouse as a means of detecting persisting *M. leprae,* and an estimate of the number of persisting organisms.

Materials and methods. Specific-pathogen-free pregnant female rats were purchased from the Charles River Breeding Laboratory, Wilmington, Massachusetts, U.S.A. Within 24 hr of birth, newborn rats were anesthetized by immersion in crushed ice for 4 min, after which thymectomy was performed with the assistance of a dissecting microscope. The upper two-thirds of the sternum was removed, exposing the thymus, which was then freed from its attachment to the anterior mediastinum and removed by blunt dissection. The area was then examined under higher magnification, and any remaining thymic tags were removed, after which the incision was closed with 6-0 silk sutures and sprayed with flexible collodion. The newborn rats were then warmed by means of a 60 W incandescent lamp until normal respiration resumed, after which they were returned to their mothers. NTR were weaned at four weeks of age, after which they were housed under conventional conditions, save that the cages were equipped with filter caps. Despite depletion of both circulating leukocytes and thymus-derived lymphocytes ([4]), NTR did not develop wasting disease, and survived for 2–3 years.

Eighteen previously untreated patients with lepromatous leprosy were randomly allocated to treatment by regimen A—a single initial 1500-mg dose of rifampin, together with 100 mg dapsone daily—or regimen C—weekly 900-mg doses of rifampin, together with 100 mg dapsone daily. Biopsies were performed after 2–6, 7–9, 14–16, and 28–30 days, *M. leprae* were recovered from the biopsy specimens, intact BALB/c mice were inoculated in both hind foot pads with 5000 organisms per foot pad, and NTR were inoculated in both hind foot pads, usually with 10^5–10^7 *M. leprae.*

M. leprae were harvested from the inoculated foot pads of BALB/c mice after one year. When *M. leprae* were found to have multiplied in mice—i.e., the harvest yielded at least 10^5 organisms per foot pad—the original suspension was considered to have contained viable *M. leprae* in a proportion not smaller than 1:5000. *M. leprae* were harvested from NTR one year or longer after inoculation. The organisms were considered to have multiplied if harvest yielded

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at least 10-fold the number of organisms that had been inoculated. On many occasions, after the harvest had yielded organisms, but fewer than 10-fold the number inoculated, *M. leprae* were subinoculated into the hind foot pads of immunologically intact mice, 5000 organisms per foot pad. Multiplication of the *M. leprae* in passage mice was also accepted as evidence that the original inoculum had included viable organisms.

Results. The biopsy specimens obtained before treatment from all 18 patients contained *M. leprae* capable of multiplying in normal mice. Results were reported of the study of 66 specimens obtained during treatment from the 18 patients. In the case of only one specimen did the organisms multiply in mice; therefore, the proportion of viable *M. leprae* recovered from the remaining 65 specimens was smaller than 1:5000.

The results of study of the 66 specimens by inoculation of $\geq 10^5$ *M. leprae* (except for the two specimens of patient 19, from which insufficient numbers of organisms were recovered) into the foot pads of NTR, are presented in Table 1. These results suggest that viable (persisting) organisms were found with considerable frequency—in 33 of 66 specimens, representing 13 of the 18 patients.

The two regimens appeared to differ in terms of efficacy. However, inspection of the data of Table 1 suggests that the difference of effectiveness between the two regimens may be only apparent. The biopsy specimens obtained from the patients allocated to regimen C yielded larger numbers of organisms, permitting larger inocula. Thus, the mean number of organisms inoculated per specimen for regimen C patients (5.35×10^7) was more than twice as large as that for regimen A patients (2.50×10^7). This is consistent with the detection of persisters in twice as many regimen C as regimen A patients, and in twice as many regimen C as regimen A specimens.

An additional analysis of the data of Table 1 is summarized in Table 2. Assuming little change of the proportion of viable *M. leprae* between the first and the last biopsies for each patient, the results of inoculation of NTR were summed for each patient. In particular, the total number of organisms inoculated into NTR was calculated for each patient by summing the numbers of organisms inoculated into each NTR that was later harvested. The number of viable organisms included within this total number of organisms was the sum of the most probable number (MPN) of viable *M. leprae* for each specimen in which viable organisms were detected, calculated by means of the equation of Halvorson and Ziegler ([7]), assuming that a 10-fold larger inoculum would have infected all of the foot pads inoculated, whereas an inoculum containing only one tenth of the number of organisms actually inoculated would not have infected any of the foot pads. The proportion of viable organisms detected in each patient was then calculated by dividing the number viable by the total number inoculated. The total number of organisms inoculated for each regimen, and the number of viable *M. leprae* included within this number were calculated by summing the corresponding numbers for each patient, and the proportion of viable organisms for each regimen was calculated by dividing the number viable for the regimen by the total number of organisms inoculated for that regimen. In fact, as shown in Table 2, the mean proportion of viable organisms was virtually identical for the patients of both regimens.

The small proportions of viable *M. leprae* observed are striking. Assuming that the average San Francisco patient had a BI = 4 (the BI was not recorded), and assuming the equivalence of a BI of 4 with a total bacterial population of 10^{11} ([10]), the average population of persisters may be calculated to be $6.33 \times 10^{-8} \times 10^{11} = 6.33 \times 10^3$.

Discussion. This small clinical trial, designed to compare directly the sensitivities of the normal mouse and the NTR to small proportions of viable *M. leprae,* demonstrated conclusively the greater sensitivity of the NTR, as has already been reported ([5, 6]). These results also permit a comparison, albeit indirect, of the sensitivities of the T900R mouse and the NTR.

The capacity of the T900R mouse to detect persisting *M. leprae* had been measured in a number of clinical trials, the most recent being the THELEP controlled clinical trials in Bamako and Chingleput ([14, 15]). As

TABLE 1. *Detection of persisting* M. leprae *by inoculation of NTR.*

Patient no.	No. days	No. AFB per specimen ($\times 10^7$)	Inoculum per foot pad ($\times 10^6$)	No. NTR foot pads		No. mouse passages	
				Total	Positive	Total	Positive
			Regimen A				
1	5	1.32	0.38	4	0	0	—
	9	0.23	0.07	4	0	0	—
	15	0.07	0.02	4	0	0	—
2	3	1.48	0.58	6	0	0	—
			0.058	6	0	0	—
	8	0.80	0.003	4	0	0	—
	15	3.97	0.18	6	0	0	—
	29	1.04	0.46	6	0	0	—
3	3	9.33	2.6	4	0	0	—
	7	16.4	7.4	6	1	1	1
			0.74	6	3	2	1
	14	9.63	2.6	4	0	2	0
			0.52	6	0	0	—
	28	11.0	2.97	6	0	1	0
			0.50	6	0	1	0
4	2	12.9	5.0	6	0	2	0
			0.50	6	0	0	—
	7	15.9	10.0	6	0	1	0
			1.0	6	0	0	—
	14	7.06	1.0	6	0	1	0
			0.50	4	0	0	—
	28	7.61	2.5	6	0	1	0
			0.50	6	0	1	0
5	3	5.51	1.5	6	0	2	0
			0.50	6	0	0	—
	7	0.47	0.10	4	0	0	—
	14	2.98	1.0	6	0	1	0
			0.10	6	0	0	—
	28	4.73	2.6	4	0	0	—
			0.50	8	0	0	—
6	3	4.12	1.1	6	5	3	3
			0.11	4	3	1	1
	7	41.1	13.2	6	1	1	1
			1.32	6	4	1	1
	15	30.2	12.5	6	0	3	0
			1.25	6	3	1	1
	28	41.1	22.2	6	1	2	1
			2.22	6	3	3	2
8	8	5.22	1.2	6	0	1	0
			0.12	6	0	0	—
	16	7.67	2.5	6	0	1	0
			0.25	6	0	0	—
	28	3.20	1.8	6	0	0	—
			0.18	4	0	0	—
9	6	48.0	7.4	6	3	3	3
			0.74	6	0	1	0
	9	29.0	3.9	6	3	1	1
			0.39	6	0	0	—
	16	32.0	5.9	6	2	2	2
			0.59	6	0	1	0
10	3	NR*	0.23	6	1	1	0
	7	NR	3.5	4	1	2	1
			0.35	6	0	0	—
			Regimen C				
12	3	13.7	3.53	6	0	1	0
			0.50	6	1	1	1
	8	11.7	4.22	6	0	0	—
			0.422	6	0	0	—

TABLE 1. *Continued.*

Patient no.	No. days	No. AFB per specimen ($\times 10^7$)	Inoculum per foot pad ($\times 10^6$)	No. NTR foot pads		No. mouse passages	
				Total	Positive	Total	Positive
	13	18.3	6.2	6	0	1	0
			0.62	6	0	0	—
	29	10.2	3.31	4	0	0	—
			0.50	6	0	0	—
13	4	27.7	15.3	6	3	3	3
			1.53	6	3	2	1
	7	9.87	50	6	0	2	0
			5.0	6	3	3	3
	14	16.4	9.55	6	1	3	1
			1.0	6	4	3	3
	28	27.0	13	6	2	3	2
			1.3	6	0	0	—
14	4	2.61	1.0	10	8	3	2
	7	3.18	1.63	6	4	1	1
			0.163	6	4	1	1
	14	1.67	2.73	6	0	0	—
			0.50	6	0	0	—
	28	0.92	0.30	12	0	0	—
15	2	69.1	10	6	3	3	3
			1.0	6	2	3	2
	7	63.2	10	6	2	3	2
			1.0	6	1	2	1
	14	72.7	30	6	2	3	2
			10	6	2	2	1
			1.0	6	1	1	1
16	3	20.3	1.0	8	0	0	—
			0.10	6	0	0	—
	7	17.8	3.5	6	1	2	1
			0.50	6	0	1	0
	14	19.0	2.6	6	0	1	0
			0.50	6	0	1	0
	28	17.7	2.4	6	0	0	—
			0.50	6	0	1	0
17	5	36	19	6	1	2	1
			1.9	6	5	2	2
	7	36	21	6	1	3	1
			2.1	6	1	1	1
	14	21	12	6	3	3	3
			1.2	6	1	2	0
	28	35	19	6	1	1	1
			1.9	4	0	0	—
18	6	4.4	1.5	6	2	2	2
			0.15	6	0	0	—
	9	2.8	0.55	6	0	0	—
	16	7.9	1.3	6	0	1	0
			0.13	6	0	0	—
	30	25	6.4	6	0	2	0
			0.64	6	0	0	—
19	6	NR	0.062	6	6	0	—
	7	NR	0.015	6	6	0	—
20	3	6.0	1.6	4	4	0	—
			0.16	4	3	1	1
	7	13	3.0	4	0	0	—
			0.3	6	4	1	1
	14	5.6	1.4	6	1	0	—
			0.14	6	0	0	—
	29	7.4	1.4	6	0	0	—
			0.14	6	0	0	—

* Not recorded.

TABLE 2. *Summary of data on detection of persisting* M. leprae *in NTR.*

Patient no.	No. specimens		No. AFB		Proportion viable ($\times 10^{-8}$)
	Total	Positive	Total ($\times 10^7$)	Viable	
			Regimen A		
1	3	0	1.56	<1	<6.4
2	4	0	0.756	<1	<13
3	4	1	9.36	5.6	6.0
4	4	0	12.6	<1	<0.79
5	4	0	3.34	<1	<3.0
6	4	4	32.3	32.1	9.9
8	3	0	3.59	<1	<2.8
9	4	4	14.8	11.9	8.0
10	2	2	1.75	3.3	19
All regimen A	32	11	80.1	52.9	6.6
			Regimen C		
12	4	1	11.0	1.86	1.7
13	4	4	58.0	21.2	3.7
14	4	2	4.34	20.1	46
15	4	4	44.4	19.6	4.4
16	4	1	6.86	1.86	2.7
17	4	4	46.5	21.4	4.6
18	4	1	6.44	2.58	4.0
19	2	2	0.046	28.8	6200
20	4	3	3.93	21.7	55
All regimen C	34	22	182	139	7.6

stated elsewhere in this collection of papers ([15]), persisting *M. leprae* were detected in about 9% of 468 specimens studied in the course of the Bamako and Chingleput trials, a proportion much smaller than that found to contain persisters in this small trial in San Francisco. The difference between the results of the San Francisco trial and those of the much larger THELEP trials cannot have resulted from treatment by regimens differing greatly in efficacy. Just as no differences could be detected between regimens A and C of the San Francisco trial, so no differences were detected among the regimens tested in the THELEP trials, in terms of the frequency with which persisters were detected. Moreover, regimen A of this trial is identical with regimen C of the THELEP trials.

Alternatively, the different frequencies of persisting *M. leprae* detected in San Francisco and the THELEP trials could perhaps be explained by the different durations of treatment after which patients were biopsied; the longest duration in San Francisco was one month, whereas the shortest duration in the THELEP trials was three months. However, no difference was observed from interval to interval in the THELEP trials ([15]). Thus during therapy continued killing of *M. leprae* and removal of dead bacilli appear to parallel one another, leaving the proportion of viable *M. leprae* in tissues relatively constant. Both the THELEP and San Francisco trials confirm earlier trials of rifampin, in the course of which the rate at which *M. leprae* were killed was measured by inoculation of normal mice; these trials demonstrated the profound bactericidal effect of rifampin virtually immediately after the first dose of the drug ([1, 8, 9, 11–13]).

The likely explanation of the difference between the results of the San Francisco trial and those of the THELEP trials resides in the greater sensitivity of the NTR, compared to that of the T900R mouse, in terms of the ability to detect very small proportions of viable *M. leprae.* This greater sensitivity may be explained by a greater degree of immunosuppression. Inoculation of larger numbers of *M. leprae* ($\geq 10^5$) into the foot pads of rodents induces an immune response that limits multiplication of the organisms. Soon after the number of *M. leprae* reaches 10^6 per foot pad of the BALB/c

mouse, multiplication ceases and killing of the *M. leprae* begins ([16]). The NTR is only partially immunosuppressed; higher inoculation of 10^7 organisms into one hind foot pad reduced multiplication of the organisms following a later challenge in the contralateral foot pad ([2]). In addition, inoculation of NTR with 10^7 *M. leprae* obtained from skin-biopsy specimens of patients undergoing initial chemotherapy did not yield multiplication more frequently than did inoculation with 10^6 organisms ([6]). However, it appears unlikely indeed that NTR are more profoundly immunosuppressed than are T900R mice.

As shown by the results of the San Francisco trial, the frequency of persisters after treatment by a rifampin-containing regimen was about 7×10^{-8}. This proportion is too small to be detected by inoculation of normal mice with 5000 *M. leprae* per foot pad, except as the merest coincidence; this may explain the multiplication of *M. leprae* that occurred in one of the 66 specimens in the course of the San Francisco trial. The greater sensitivity of the NTR probably resides in the fact that a much larger inoculum may be employed; because of the larger size of the foot of the rat, one may inoculate volumes as large as 0.5 ml into the foot pad of the NTR, whereas the inoculum for a mouse is usually administered in a volume of 0.03 ml. In fact, because of the larger foot pad, the NTR is probably more useful than nude mouse for detection of persisters; the nude mouse is more profoundly immunosuppressed, but the number of organisms that can be inoculated into the foot pad of the nude mouse is limited by the volume tolerated. If the organisms were more concentrated, it might be possible to inoculate nude mice with larger numbers of *M. leprae*; however, it may not be possible to concentrate the organisms without reducing their capacity to multiply.

The proportion of viable *M. leprae* in specimens obtained from patients with previously untreated lepromatous leprosy may frequently be smaller than 1:100 (Gelber, unpublished results; Grosset, personal communication). Therefore, a single dose of rifampin, which reduces the proportion of viable organisms to 1 per 10^5–10^7 *M. leprae,* has killed 99.9–99.999% of the viable organisms originally present. This is consistent with the observation that, among the *M. leprae* recovered from specimens of rifampin-treated patients, viable organisms are not detected (by multiplication) in normal mice inoculated with 5000 organisms per foot pad, are detected irregularly in T900R mice inoculated with 10^5 organisms per foot pad, and may be detected more frequently in NTR inoculated with 10^6–10^7 *M. leprae* per foot pad.

A very important result of the San Francisco trial is that it has verified the findings of the THELEP trials, with respect to the mean numbers of viable *M. leprae* surviving treatment. The number of organisms persisting in the average patient is calculated here to be approximately 6000, a number not greatly different from the 50,000–250,000 estimated from the results of the THELEP trials.

In summary, it appears that, virtually immediately after treatment is begun by a regimen that includes rifampin, the great majority of the viable *M. leprae* are killed. The proportion of surviving organisms is so small that viable organisms cannot be detected by inoculation of normal mice with 5000–10,000 organisms per foot pad, are detected in only a minority of instances by inoculation of T900R mice with 10^5 *M. leprae* per foot pad, and are much more readily detected by inoculating the foot pads of NTR with 10^6–10^7 organisms. This proportion appears to change very little during the first month of treatment, and probably even during the first 1–2 years of treatment ([14, 15]). Finally, the absolute number of persisting *M. leprae* harbored by the average patient after treatment with a regimen including rifampin has been initiated appears indeed to be very small, no larger than 2.5×10^5, and probably as small as 6×10^3.

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