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Relationship Between T-Cell Population in Neonatally Thymectomized Lewis Rats and Susceptibility to Infection With *Mycobacterium leprae*[1]

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We have developed the neonatally thymectomized Lewis rat (NTLR) as a model of multibacillary leprosy and have utilized it both for chemotherapy studies and as a means of detecting persisting viable organisms in the tissues of patients undergoing therapy ([5, 6, 7]). However, we have found that even though the NTLR are thymectomized within 18 hr of birth, the response of some of them to *M. leprae* infection is similar to that of intact rats. Yet they show no evidence of residual thymus when they are killed at one to two years of age. Since NTLR rarely develop wasting disease, it is probable that they may be born with a certain—and probably varying—degree of immunological competence, which may be related to an observed variation in the gestation period. It is possible that those rats born more than 21 days after conception develop a greater degree of immunological competence by the time they are born, and therefore neonatal thymectomy has little or no effect. Because it is important to eliminate these immunologically competent animals from experiments, we examine ear snips from intravenously inoculated NTLR for the presence of *M. leprae*. We have found that if the ear contains at least 10^5 *M. leprae*, the animal will have a well-developed, disseminated infection. Animals with fewer organisms are discarded. However, these procedures are time consuming and expensive since generalized infection does not occur in the NTLR until at least one year after inoculation. We have therefore attempted to develop an *in vitro* test for immunosuppression of NTLR by determining whether a relationship exists between the remaining population of T-cells and the susceptibility of these animals to generalized infection with *M. leprae*. If such a relationship existed, it would be possible to eliminate these animals at the time of weaning.

MATERIALS AND METHODS

Animals. Pregnant Wistar-Lewis rats were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. Purportedly, these animals were inbred, although this now appears to be questionable ([3]). However, Charles River Laboratories have assured us that their barrier-raised foundation stock are indeed brother-sister mated Lewis rats, although some genetic drift has occurred in their production stock. As a result, about 10% of the production stock reject grafts from the foundation stock.

Thymectomy. All rats were thymectomized between 5 and 16 hr after birth by the method previously described by us ([6]).

Mycobacterium leprae. The strain of *M. leprae* used in these experiments was obtained from Dr. C. C. Shepard, Center for Disease Control, Atlanta, Georgia, who had isolated it from a skin biopsy specimen obtained from a patient with lepromatous leprosy. It has been maintained in our laboratory since 1968 by passage through rats. The methods of inoculation, processing of infected tissues, and counting of *M. leprae* have been described previously ([6, 10, 11]).

Antithymocyte globulin. A highly specific, absorbed antithymocyte globulin was labeled with fluorescein isothiocyanate (FITC) and used in a direct fluorescent antibody test to determine whether there was a correlation between the number of remaining

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T-cells and susceptibility to *M. leprae* infection. The method for producing the labeled antibody was as follows. The starting material was 50 ml of rabbit anti-rat thymocyte serum obtained from A. L. Monaco, Harvard Medical School, Boston, Massachusetts. It was prepared in his laboratory by the method of Gray, *et al.* [8]. It had a cytotoxicity titer of 1:1024 against Lewis rat thymocytes and 1:512 against rat bone marrow cells, as determined by the trypan blue dye exclusion test. The serum was adsorbed twice with packed rat red blood cells (RBC) overnight at 4°C. The γ-globulin fraction was then precipitated with saturated $(NH_4)_2SO_4$ at a final concentration of 40%. After centrifugation the precipitate was dissolved in 50 ml of phosphate buffered saline (PBS), and the precipitation procedure was repeated. The protein concentration was adjusted to 1% with a refractometer. The γ-globulin solution was dialyzed at 4°C for 3 days against several changes of PBS and then labeled with FITC by the method of Clark and Shepard [4]. The labeled globulin was then absorbed twice with fetal rat liver homogenate, insolubilized rat serum, and insolubilized rat kidney [1,2]. Finally, the solution was clarified by passage through a Sephadex G 25 column (1 part FITC-labeled antibody to 2.5–3 parts of Sephadex gel) and sterilized by Millipore filtration. The cytotoxicity titer of the final product was 1:16 for thymocytes and <1:2 for bone marrow cells. A 1:5 dilution of the conjugated antibody stained 96.3% (±3.8) of thymocytes and 0.1–0.8% of bone marrow cells.

Direct immunofluorescent test for T-cells. Dextran T 500 (2 ml of a 4.5% solution) in Eagle's minimum essential medium (MEM) containing 1 mg/ml heparin (sodium salt) was drawn into a 3 ml plastic syringe fitted with a 1.5-inch 19 gauge needle. Blood (0.1 to 0.3 ml) from a tail vein of an anesthetized rat was drawn into the syringe and thoroughly mixed with the dextran solution. The syringe, needle end up, was incubated at room temperature for 1.5 to 2 hr, or until most of the RBC had sedimented. The syringe was refitted with an 18 gauge needle and a 45 cm length of Tygon tubing, the other end of which was placed in a 15 ml centrifuge tube. The piston was then slowly pushed up until all of the supernate containing the leukocytes was transferred to the centrifuge tube. MEM (8 ml) was added to the suspension, which was then centrifuged at 200 × *g*. The supernate was discarded and the pellet was resuspended in 2 ml of Tris-NH_4Cl buffer and incubated at 35°C for 5 min to lyse the RBC. The cells were again suspended in MEM and the procedure was repeated until no RBC were present. The white blood cells (WBC) were resuspended in 0.5 ml of a 1:15 dilution of the fluorescein conjugated antithymocyte globulin and incubated at 5°C for 50 min. MEM (9.5 ml) was added, and the suspension was centrifuged at 200 × *g*. The supernate was discarded, and the cells were resuspended in no more than 0.2 ml of MEM and then mounted on a slide. The cells were counted in a Zeiss photomicroscope under darkfield illumination from an ultraviolet source. The exciter filter was BG 12 and the barrier filter was 44. Positive staining consisted of a bright, uneven fluorescent band around the periphery of the cell and bright fluorescent dots that appeared to be within the cell. Occasionally positive cells were aggregated.

Lymphocyte transformation studies. Spleens were removed aseptically from Lewis rats approximately 12 months old. Single cell suspensions were prepared by carefully teasing in RPMI 1640 medium containing 10% fetal bovine serum. The resulting suspensions were centrifuged at 50 × *g* for 5 min to sediment large clumps and erythrocytes. The supernatant was centrifuged at 100 × *g* and the lymphocytes were resuspended in 5 ml of medium. The lymphocyte suspension was diluted to 3×10^6 cells per ml, and 1 ml aliquots were transferred into round-bottom glass test tubes. A 50 μl aliquot of medium containing concanavalin A (Sigma Chemical Co., St. Louis, Missouri), to give a final concentration of 25 μl per ml, was added to three tubes and 50 μl of medium was added to a fourth (control) tube. The cultures were incubated for 48 hr at 37°C in an atmosphere of 5% CO_2, after which 1 μCi of tritiated thymidine was added to each culture. After an additional 18 hr of incubation, the lymphocytes were washed three times in PBS and finally in 5% trichloroacetic acid (TCA). The TCA

TABLE 1. *Relationship between T-cell populations in peripheral blood of neonatally thymectomized Lewis rats and their susceptibility to foot pad infection with* M. leprae.

Group[a]	Total WBC/mm^3	Percent T-cells	Age of NTLR[b] (days)		AFB/foot pad[c,d]
			At inoculation	At death	
A	N.D.[e]	0	37	369	1.04×10^9
	5300	0	36	276	4.50×10^6
	3450	1	34	425	3.58×10^6
	5750	0	40	375	4.22×10^8
	7800	0	42	425	1.47×10^9
	5300	3	40	427	5.29×10^9
B	N.D.	10	37	426	1.52×10^6
	6950	5	43	426	1.67×10^7
	7650	8	40	231	6.88×10^7
	3500	8	41	276	1.41×10^8
	4300	8	41	367	5.35×10^7
C	3350	19	36	276	3.14×10^7
	4300	31	36	367	2.15×10^6
	5400	15	41	426	2.50×10^8
	5400	18	36	429	8.13×10^8
D	11,473[f]	45	—	—	N.D.[g]

[a] Group A, 0–3% T-cells; Group B, 5–10% T-cells; Group C, 15–31% T-cells; Group D, intact—not inoculated.
[b] NTLR were inoculated with 1×10^4 *M. leprae*/foot pad.
[c] Average of both left and right hind foot pads.
[d] The variance among the three groups was determined by least-squares means. The p values for A compared to B, B to C, and A to C were 0.2302, 0.6435 and 0.5019 respectively. Thus there are no significant differences among the three groups.
[e] N.D. = Not done.
[f] WBC count on intact rats represents an average for 10 animals.
[g] We have previously determined that the ceiling of multiplication of AFB in the foot pads of intact Lewis rats is no greater than 2.8×10^6 per foot pad (9).

precipitates were collected on 25 mm Millipore filters and counted in a Searle Mark III liquid scintillation counter. Lymphocyte stimulation indices were calculated as the ratio of the mean count of three concanavalin A cultures to the count of the control culture.

RESULTS

Relationship between T-cell populations in peripheral blood of NTLR and their susceptibility to infection with *M. leprae*. The WBC count on blood from 28 NTLR 5 to 7 weeks old averaged 5226 ± 1337 (S.D.) mm^{-3}, and the number of T-cells ranged between 0 and 31% (mean 9.11%). The WBC count on five intact Lewis rats averaged 11,473 ± 1096, and the T-cell population averaged 44.7 ± 1.16%.

To determine whether there was a correlation between the percentage of circulating T-cells and susceptibility to infection with *M. leprae*, we separated 15 of the NTLR into three groups (4–6 animals per group): those with 0 to 3% T-cells (Group A), 5 to 10% T-cells (Group B), and 15 to 31% T-cells (Group C). All of the animals were inoculated in both hind foot pads with 1×10^4 *M. leprae*. The results are shown in Table 1. There were no significant differences among the three groups. Although the highest counts of acid-fast bacilli (AFB) were found in the group with the lowest number of circulating T-cells, there were two animals in that group whose AFB counts were no greater than those generally found in intact rats. One of the NTLR in Group C had 18% T-cells, yet the AFB in its hind foot pads averaged 8.13×10^8.

The circulating T-cells, determined on a second group of 13 NTLR 4 to 6 weeks old, ranged from 2 to 21%. When they were approximately 6 weeks old, they were inoculated intravenously in a tail vein with 1.94×10^7 *M. leprae* and arbitrarily divided into three groups: those with 2 to 4% T-cells, those with 5 to 8% T-cells, and those with 11 to 21% T-cells. They were

TABLE 2. *Relationship between T-cell populations in peripheral blood of neonatally thymectomized Lewis rats and their susceptibility to intravenous infection with* M. leprae.

Group	Percent T-cells	Duration of infection[a] (days)	AFB/hind foot pad[b]	AFB/front foot pad[b]	AFB/ear[b]	AFB/nose	AFB/tail[c]
A	3	410	4.93×10^6	8.67×10^5	1.22×10^6	1.45×10^6	1.27×10^9
	2	589	6.94×10^7	N.D.	6.94×10^4	N.D.	1.35×10^6
	2	713	1.87×10^8	1.68×10^8	1.88×10^7	N.D.	2.15×10^9
	2	782	4.19×10^8	9.54×10^7	3.53×10^7	1.48×10^8	1.02×10^{10}
	4	963	4.74×10^5	7.32×10^4	5.75×10^4	$<10^4$[d]	3.43×10^6
B	6	503	N.D.	N.D.	1.68×10^7	N.D.	N.D.
	8	651	6.14×10^4	N.D.	3.22×10^4	N.D.	8.53×10^7
	7	710	3.62×10^6	1.07×10^6	8.32×10^5	N.D.	3.53×10^8
	5	816	1.56×10^6	7.70×10^4	$<10^4$	1.84×10^5	4.90×10^6
C	14	434	3.15×10^5	1.63×10^4	3.38×10^4	$<10^4$	1.20×10^7
	21	556	1.44×10^7	N.D.	6.97×10^5	N.D.	5.50×10^8
	11	680	2.61×10^5	1.15×10^5	$<10^4$	$<10^4$	4.91×10^5
	11	856	1.21×10^6	1.29×10^5	9.73×10^4	$<10^4$	6.78×10^6

[a] Inoculated intravenously with 1.94×10^7 *M. leprae* and died on the indicated day after inoculation.
[b] Individual counts made on each foot pad and ear. The figures represent average number of *M. leprae* per foot pad or ear.
[c] The variance between the three groups was determined by least-squares means. The p values for A compared to B, B to C and A to C were 0.6489, 0.5793 and 0.2731 respectively. Thus there were no significant differences among the three groups.
[d] $<10^4$ AFB = No organisms seen in 60 fields.

then allowed to live out their life span, at the end of which time *M. leprae* were enumerated in various organs. The results, shown in Table 2, were generally similar to those found in the foot pad inoculated NTLR shown in Table 1, and again there were no significant differences among the three groups. The greatest numbers of organisms were found in the group that had 2 to 4% circulating T-cells. The tails of three of the five NTLR in this group contained between 1.27×10^9 and 1.02×10^{10} *M. leprae*. However, the remaining two tails in this group had almost the lowest numbers of *M. leprae* among the 13 NTLR investigated. The NTLR that had 21% circulating T-cells (the highest percentage found among these animals) had 5.50×10^8 *M. leprae* in the tail, which was the fourth highest among the 13 NTLR. In spite of the

TABLE 3. *Results of intravenous inoculation of nonthymectomized Lewis rats with* M. leprae.

Inoculum	Duration of infection (days)	AFB/hind foot pad[a]	AFB/front foot pad[a]	AFB/ear[a]	AFB/nose	AFB/tail
1.65×10^7	293	$<10^4$[b]	$<10^4$	$<10^4$	$<10^4$	3.95×10^5
	376	$<10^4$	$<10^4$	$<10^4$	$<10^4$	1.31×10^5
	431	$<10^4$	$<10^4$	4.00×10^4	$<10^4$	4.26×10^5
	687	1.20×10^5	$<10^4$	$<10^4$	$<10^4$	$<10^4$
	734	$<10^4$	$<10^4$	$<10^4$	$<10^4$	1.25×10^5
1.89×10^7	903	7.63×10^5	2.99×10^5	$<10^4$	$<10^4$	1.57×10^5
	963	1.20×10^5	1.07×10^5	$<10^4$	$<10^4$	$<10^4$
	990	2.50×10^4	$<10^4$	$<10^4$	$<10^4$	$<10^4$
	991	7.28×10^5	1.68×10^5	$<10^4$	$<10^4$	1.54×10^5

[a] Individual counts made on each foot pad and ear. The figures represent the average number of *M. leprae* per foot pad or ear.
[b] $<10^4$ AFB = No organisms seen in 60 fields.

variation in the *M. leprae* infection among the NTLR, all of the animals were infected to some degree. This is in contrast to the results obtained after intravenous inoculation of intact Lewis rats with *M. leprae*, as shown in Table 3. It is apparent that even in animals infected more than 2.5 years earlier, only a limited infection was found in the tails and foot pads. In only one instance were organisms found in the ear, and in no instance did we find *M. leprae* in the nose.

Lymphocyte transformation in NTLR. Although these studies are clearly of no practical value with respect to detecting immunocompetent NTLR, they were carried out to determine whether there was any correlation between T-cell activity and susceptibility to *M. leprae* infection. We therefore determined the response of cultured splenic lymphocytes to the T-cell mitogen concanavalin A for three groups of rats: normal Lewis controls; NTLR in which ear biopsies taken approximately 12 months after intravenous inoculation with *M. leprae* were positive for AFB (these were designated NTLR+); and NTLR in which ear biopsies taken 12 months after intravenous inoculation with *M. leprae* were negative for AFB, and which were therefore assumed to be inadequately immunosuppressed (these were designated NTLR−). As shown in Table 4, both groups of NTLR showed significantly reduced stimulation indices compared with controls, in which the mean was 33.5 (±5.33 S.E.M.) and the range was 12 to 77. In the group designated NTLR+, the mean stimulation index was 3.3 (±0.51), with indices ranging from 1.9 to 4.85. The group designated NTLR− had a stimulation index of 6.5 (±1.80), with indices ranging from 0.9 to 20.1. Thus, although the mean stimulation index of the heavily infected group (NTLR+) was lower than that of the group that showed no evidence of generalized infection (NTLR−), the difference was not significant and there was considerable overlap of indices between the two groups.

TABLE 4. *Relationship between lymphocyte transformation in NTLR and susceptibility to* M. leprae *infection.*[a]

Control rats (N = 12)	NTLR+[b] (N = 7)	NTLR−[c] (N = 13)
33.5 (±5.33)	3.3 (±0.51)	6.5 (±1.80)

[a] Values are expressed as mean stimulation index (± standard error of the mean). Background incorporation of tritiated thymidine was essentially the same in all three groups.

[b] NTLR+ = Those NTLR in which AFB were found in an excised ear, indicating a well-developed, generalized infection.

[c] NTLR− = Those NTLR in which no AFB were found in an excised ear, indicating a poor infection.

DISCUSSION

Neonatal thymectomy of Lewis rats resulted in a severe depletion in both the total number of circulating WBC and the thymus-derived lymphocytes. The WBC count in intact Lewis rats was more than twice as great as that found in NTLR, and the average number of circulating T-cells in the blood of normal rats was about fivefold greater than that in NTLR. Although there was little variation in the percentage of circulating T-cells among normal rats, there was a great variation in the circulating T-cells of NTLR. However, in no instance did we find NTLR with a normal number of T-cells. Nevertheless, it was not possible to demonstrate a close correlation between circulating T-cells and susceptibility of NTLR to infection with *M. leprae*. In general, those NTLR that had the lowest number of circulating T-cells in the blood had the highest number of *M. leprae* in the foot pads after inoculation in the foot pads. However, in these groups there were also animals that responded to infection much like normal rats. The same was true of intravenously inoculated NTLR in that the greatest number of *M. leprae* were found in the tails of those animals that had the lowest number of circulating T-cells, but some of the lowest counts were also found in these groups. It is noteworthy, however, that a generalized infection with *M. leprae* developed in all of the intravenously inoculated NTLR, whereas the infection was quite limited in the intact rats.

The results of the lymphocyte transformation tests basically confirmed the tests using direct visualization of the T-cells. The mean stimulation index of splenic lymphocytes from normal rats was about five to ten times greater than those for NTLR. However, with this method it was still not possible to distinguish between NTLR with

a well developed, generalized infection and those with a poorly developed infection. In fact, one NTLR that had no AFB in either ear and only 2.19×10^6 AFB in the tail had a stimulation index of 2.06. Another NTLR that had 1.77×10^7 *M. leprae* in one ear and 1.09×10^9 organisms in the tail had a stimulation index of 4.85.

The results of these experiments reveal the great immunologic variation that exists among NTLR. All of the NTLR had some degree of immunosuppression, as indicated by the great depletion of circulating T-cells and the reduction in the ability of the lymphocytes to transform in the presence of concanavalin A. The fact that there appeared to be little or no relationship between T-cell depletion and susceptibility to infection suggests the possibility of a dual mechanism of resistance in these animals, possibly dependent on the presence or absence of activated macrophages as well as T lymphocytes.

SUMMARY

The neonatally thymectomized Lewis rat (NTLR) is highly susceptible to infection with *M. leprae*. However, a significant percentage of NTLR respond to infection with *M. leprae* in much the same way as do intact rats, yet show no evidence of residual thymus. To determine whether there was a correlation between the number of remaining T-cells and susceptibility to infection with *M. leprae*, a direct fluorescent antibody test was performed using a highly specific, absorbed antithymocyte globulin labeled with fluorescein isothiocyanate. Both total circulating white blood cells and T-cells were significantly depressed in all NTLR examined. Although the greatest numbers of *M. leprae* were found in NTLR from the groups having the lowest percentage of circulating T-cells, these groups also contained NTLR infected with small numbers of *M. leprae*. The groups containing NTLR with the highest percentages of circulating T-cells also contained animals with both moderate and severe *M. leprae* infection. The response of cultured splenic lymphocytes from NTLR and normal rats to the T-cell mitogen concanavalin A was investigated to determine whether there was any correlation between T-cell activity and susceptibility to *M. leprae* infection. The mean stimulation index for normal rats was five to ten times greater than indices for NTLR, but there were no significant differences between NTLR with a well developed, generalized infection and those with a poorly developed infection. It was concluded that since there was no apparent relationship between T-cell depletion and susceptibility to infection with *M. leprae*, an additional, unknown mechanism was also involved.

RESUMEN

Las ratas Lewis timectomizadas durante la etapa neonatal (RLTN) son altamente susceptibles a la infección por *M. leprae*. Sin embargo, una importante proporción de las RLTN, en las cuales no se encuentran residuos tímicos, responde a la infección con *M. leprae* en forma semejante a como lo hacen las ratas intactas. Para determinar si existía alguna correlación entre el número de células T remanentes y la susceptibilidad a la infección con *M. leprae*, se efectuó una prueba de fluorescencia directa usando un anticuerpo anti-timocito específico, marcado con isotiocianato de fluoresceína. Tanto los leucocitos circulantes totales como las células T estuvieron significativamente deprimidos en todas las RLTN examinadas. Aunque los mayores números de *M. leprae* se encontraron en los animales de los grupos con los menores porcentajes de células T circulantes, estos grupos también contuvieron a RLTN infectadas con pequeños números de *M. leprae*. Los grupos conteniendo a las RLTN con los mayores porcentajes de células T circulantes también incluyeron a animales con moderada o severa infección por *M. leprae*. Para investigar si había alguna correlación entre la actividad de las células T y la susceptibilidad a la infección por *M. leprae*, se investigó la respuesta de los linfocitos esplénicos de las RLTN y de ratas normales hacia el mitógeno de células T, concanavalina A. El índice promedio de estimulación para las ratas normales fue de cinco a diez veces mayor que los índices para las RLTN, pero no hubieron diferencias significativas entre las RLTN con una infección sistémica bien desarrollada y aquellas con una infección pobremente desarrollada. Se concluyó que, puesto que no hubo una relación aparente entre la depleción de células T y la susceptibilidad a la infección por el *M. leprae*, algún mecanismo adicional hasta ahora desconocido debe estar involucrado.

RÉSUMÉ

Le rat Lewis nouveau-né thymectomisé (NTLR) est hautement susceptible à l'infection par *M. leprae*. Toutefois, un pourcentage significatif de ces rats répondent à l'infection par *M. leprae* d'une manière fort semblable à celle dont témoignent les rats intacts, alors que pourtant ils ne présentent aucun signe d'existence d'un thymus résiduel. Afin de déterminer s'il existe une corrélation entre le nombre de cellules

T qui persistent et la susceptiblité à l'infection par *M. leprae*, on a eu recours à une épreuve directe pour les anticorps fluorescents, en utilisant une globuline antithymocyte absorbée et hautement spécifique, marquée par de l'isothiocyanate de fluorescéine. Le nombre total de globules blancs circulants de même que le nombre total de cellules T, étaient significativement diminués chez tous les rats NTLR examinés. Alors que c'est chez les rats NTLR appartenant aux groupes présentant le pourcentage le plus faible de cellules T circulantes que l'on a observé le nombre le plus élevé de *M. leprae*, ces groupes contenaient cependant des rats NTLR infectés par un petit nombre seulement de *M. leprae*. Les groupes contenant des rats NTLR avec les pourcentages les plus élevés de cellules T circulantes, contenaient également des animaux qui présentaient une infection soit modérée, soit grave à *M. leprae*. La réponse de lymphocytes cultivés à partir de la rate de rats NTLR et de rats normaux, à la concanavaline A, un mitogène pour les cellules T, a été étudiée, en vue de déterminer s'il existe une corrélation entre l'activité des cellules T, et la susceptibilité à l'infection par *M. leprae*. L'index moyen de stimulation pour les rats normaux était de 5 à 10 fois plus élevé que les indices relevés pour les rats NTLR, mais on'a cependant pas observé de différences significatives entre les rats NTLR présentant une infection généralisée bien développée, et ceux qui ne souffraient que d'une infection peu développée. On en conclut sur la base d'une absence apparente de relations entre la diminution en cellules T et la susceptibilité à l'infection par *M. leprae*, qu'un mécanisme supplémentaire, et encore inconnu, est également à l'oeuvre.

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