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Dosage and Site of Entry Influence Growth and Dissemination of *Mycobacterium leprae* in T900r Mice[1]

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Many studies have reasonably documented that a large number of bacilli are shed and dispersed from the nose, mouth and ulcerating skin lesions of untreated lepromatous patients ([2, 4, 5, 6, 10]), but the route of entry of *M. leprae*, evolution of the type of disease, dissemination of the disease, and its transmission in the community is still not very clear. Several carefully controlled experimental studies on animal models have been done and these experiments in nude mice have shown that *M. leprae* can be transmitted through broken nasal mucosa ([3, 9]) and abraded skin ([8]). Our preliminary study to infect intact skin on the flanks of T900r mice, an area with a temperature relatively higher than that of feet, was not successful ([5]). To further substantiate this finding and to determine whether there was a difference in the growth and dissemination of organisms when *M. leprae* were inoculated into two different sites (footpad and flank), a comprehensive study was done and the outcomes are presented.

MATERIALS AND METHODS

A group of sixty-eight CBA mice were thymectomized at six to seven weeks of age. Three weeks later they were irradiated with 900 rad and a syngeneic bone-marrow cell transfusion as administered intraperitoneally ([11]). These immunodeficient mice (T900r mice) were now ready for inoculation with *M. leprae*. *M. leprae* suspensions were obtained from the lepromatous nodules that developed on the footpads of T900r mouse previously inoculated with *M. leprae*. The mice were randomized, 36 were inoculated intradermally in both the flanks and the other 32 in both the footpads. In each of these two groups four different concentrations of *M. leprae* inoculations were used. Inocula of *M. leprae* containing 10^7, 10^6, 10^5 and 10^4 in 0.1 ml were used in the flank group of animals. In the footpad group of animals, the different concentrations were diluted in 0.03 ml for inoculation. The animals were fed with standard food pellets and unlimited water.

One mouse from each *M. leprae* concentration group was sacrificed at the 6th, 8th and 12th month. All the remaining animals were harvested at the 15th month. The skin

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along with the subcutaneous tissue and the muscle at the injected area on both sides were dissected. Tissues from the left flanks were harvested and the AFB were counted. Tissues from the right flanks were immediately fixed in 10% buffered formalin and sent for histopathological studies. Sections of 4 μm thickness were made and stained with hematoxylin and eosin (H&E) and modified Fite Faraco stain ([7]). Autopsies were done on mice sacrificed at the end of the 15th month and clippings from the ear lobes and internal organs, such as the liver, lung, kidney, heart, and spleen, were subjected to histopathological examination. Similar techniques were followed for animals inoculated in the footpad. It was not possible to do an autopsy on one animal in the 10^4 mouse footpad group because the sacrificed animal was inadvertently discarded.

RESULTS

The *M. leprae* smear counts comparing both groups at different periods of time is given in Table 1. The harvested count showed that 10^4 in 0.1 ml inoculum did not promote growth in the mice injected in the flank, but growth was seen in all of the mice that were inoculated in the footpad with this concentration of *M. leprae*. In all other *M. leprae* concentration groups, there was growth of *M. leprae,* but it was quantitatively more in the footpad-inoculated animals than in the flank-inoculated animals. In the footpad group of animals, the multiplication was almost double that of the flank group of animals when the inoculation count was 10^5 and 10^6, but with the highest concentration of 10^7, this was not so.

Table 2 shows organs where *Mycobacterium leprae* had disseminated. A histopathological examination of the skin from the flank-inoculated group of animals showed focal granulomas occupying the dermis and subcutaneous tissues. The granulomas consisted of foamy macrophages, some with granular cytoplasm, and few lymphocytes (Fig. 1). Acid-fast stain showed macrophages containing bacilli that were mostly fragmented and granular. The ears, liver, spleen, heart, kidney showed no significant lesions, except small nonspecific mononuclear cellular inflammatory infiltration of the liver, lungs and kidneys. Dissemination of bacilli was not seen in this group of animals.

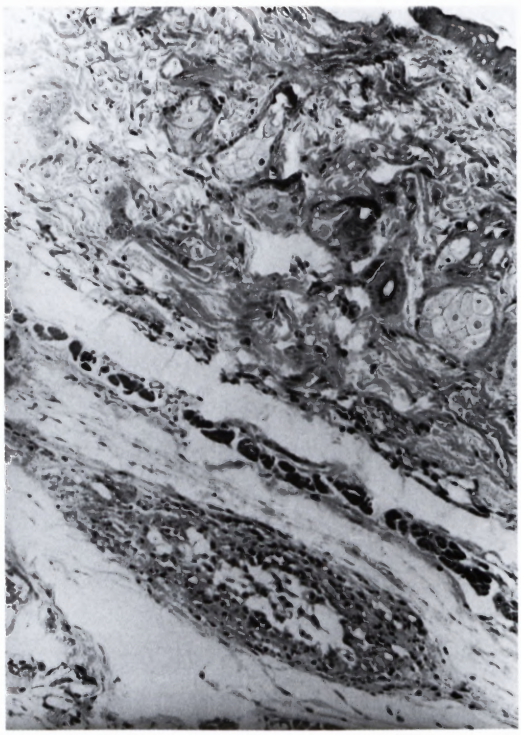

FIG. 1. Photomicrograph to show skin from the flanks with a subcutaneous granuloma composed of macrophages (H&E ×80).

A histopathological examination of footpad-inoculated mice showed granulomas in

TABLE 1. *Growth of* M. leprae *with varying inoculum dosage of* M. leprae.

Inoculum dosage of *M. leprae*	Bacterial count							
	6th month		8th month		12th month		15th month	
	Flank	Footpad	Flank	Footpad	Flank	Footpad	Flank	Footpad
10^7	1.8×10^5	8.4×10^7	7.7×10^5	1×10^8	1.5×10^5	1.4×10^7	1.3×10^7	2.3×10^8
10^6	4.6×10^5	1×10^7	5.7×10^4	3.8×10^7	5.7×10^5	8.8×10^6	3.6×10^5	6.3×10^8
10^5	1.1×10^3	1.2×10^6	Nil	1.6×10^7	6.9×10^3	2×10^8	6.6×10^2	4.8×10^8
10^4	Nil	2.2×10^5	Nil	7.3×10^6	Nil	9.9×10^7	Nil	1.5×10^8

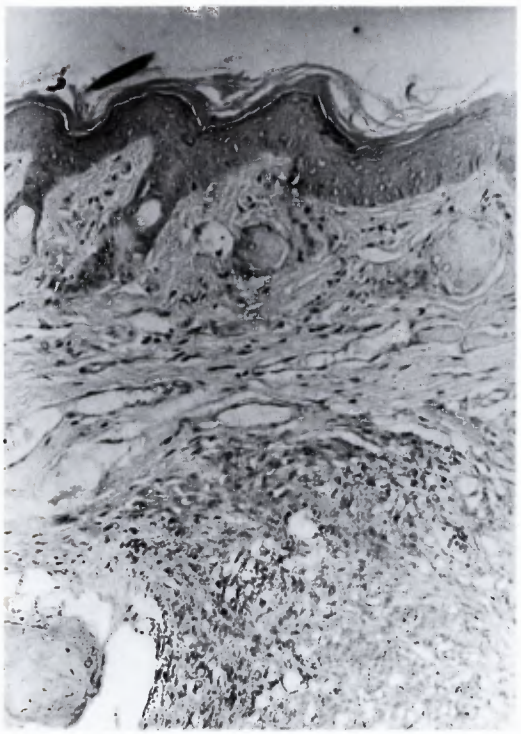

FIG. 2. Photomicrograph showing a large subepithelial granuloma in the footpads composed almost entirely of macrophages (H&E ×160).

the footpads in all the animals regardless of the *M. leprae* concentration (Fig. 2). Granulomas consisting of macrophages filled with bacilli were consistently seen at the 6th, 8th, 12th and 15th month. Autopsied tissue revealed extensive dissemination of acid-fast bacilli (AFB) forming microgranulomas in ears (Fig. 3). The bacillary index of the granuloma (BIG) was 4+ (Fig. 4). The lung and liver showed scattered AFB inside the macrophages.

DISCUSSION

When *M. leprae,* in differing concentrations, were injected intradermally into the flank skin of the mice, they were taken up by macrophages and aggregated at the site of inoculation (Fig. 1). However, these bacilli were found to be granulated and fragmented signifying degenerated bacteria. The smear count of bacilli was either less than that inoculated or zero when the concentration of *M. leprae* in the inoculum was the lowest. These features demonstrate that there was no multiplication of bacilli at the flank site. This could be due to the relative

TABLE 2. *Dissemination of* M. leprae *into various organs.*

Organs	Mice inoculated in footpad				Mice inoculated in flank			
	10^4	10^5	10^6	10^7	10^4	10^5	10^6	10^7
Ears	ND	+	+	–	–	–	–	–
Lungs	ND	+	+	–	–	–	–	–
Liver	ND	+	+	–	–	–	–	–
Spleen	–	–	–	–	–	–	–	–
Heart	–	–	–	–	–	–	–	–
Kidney	–	–	–	–	–	–	–	–

increased warmth of the flank (^{13}) compared to the footpad (^{12}). Injection of the *M. leprae* inoculum into the mouse footpad showed more than a doubling of the organism, and the organisms themselves did not display the fragmentation that was seen at the flank site. This clearly demonstrates the multiplication of *M. leprae* in the mouse footpad. However the multiplication was not uniform in all of the concentrations. The lowest (10^4) and the highest (10^7) inoculum did not demonstrate a high growth rate like that found with the 10^4 and 10^5 inoculum.

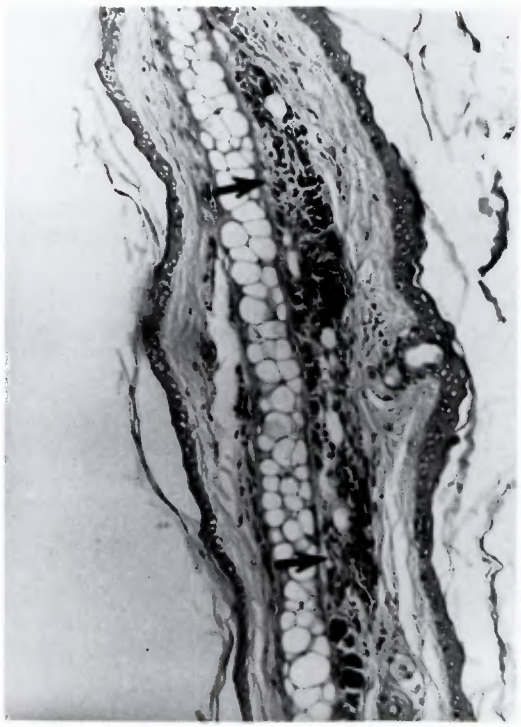

FIG. 3. Photomicrograph of cross-section of ear showing small collections of macrophages forming microgranulomas situated above the cartilage (H&E ×80).

This again clearly demonstrates that optimal levels of inoculum exist that promote growth of the organism in the mouse footpad and inoculating more bacilli than this optimal load does not yield better results.

Dissemination of *M. leprae* to the internal organs as seen in the footpad-inoculated animals was completely absent in the flank-inoculated mice. Multiplication at the site of entry and dissemination to the internal organs, when the inoculum is injected into the mouse footpad and when no growth or dissemination occurs when injected into the flank, demonstrates that local environmental changes, such as relatively cooler temperatures and an optimum dose of *M. leprae* at the site of entry, are conducive factors for the development of the disease.

In a recent study, it was found that a majority of the leprosy mono-lesions were located in the uncovered parts of the body with a special predilection for the posterior aspects of the lower extremities ([1]). In children, these sites are most vulnerable to trauma. They are also relatively cooler, and our animal study demonstrates that *M. leprae* multiply better in cooler parts, such as the footpad, and disseminate more to the ear lobes. This may explain why the first and early lesions occur in the extremities and face where the temperature is low. In mice, the entry of *M. leprae* through a relatively cooler entry point (i.e., the footpad) allows better growth of *M. leprae* locally, needs smaller doses of inoculum to promote growth, and allows dissemination of the organism to the internal organs. The site of entry and the dose of *M. leprae* may have a role in determining whether the person will be infected or not.

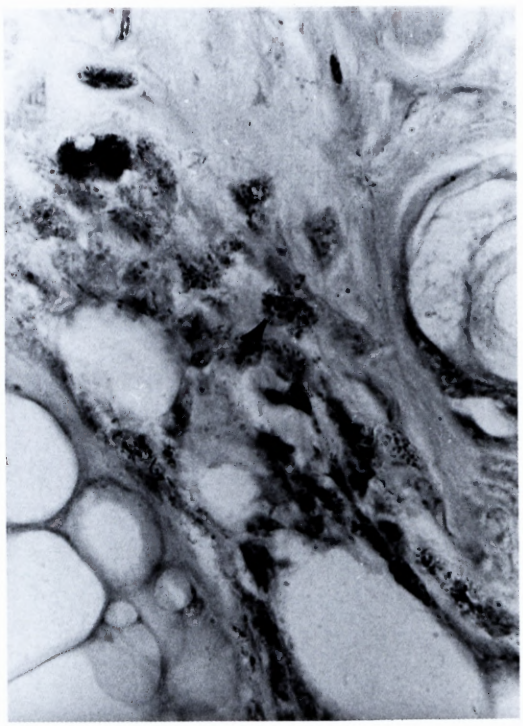

FIG. 4. Photomicrograph showing clumps of acid-fast bacilli (AFB) in macrophages shown in Fig. 3 (modified Fite Faraco stain ×800).

SUMMARY

The role of dosage of *Mycobacterium leprae* and the environment of the inoculated site, in producing leprosy lesions in immunologically-suppressed, highly-susceptible T900r mice, was investigated. Various doses of *M. leprae,* i.e., 10^7, 10^6, 10^5, 10^4, were inoculated into both flanks and footpads of two different groups of mice. The sites of inoculation were biopsied for histopathological examination and for *M. leprae* counts at the end of 6, 8 and 12 months. *M. leprae* multiplied at the infected site and disseminated to other parts of the body at all concentrations in the mice that were infected in the footpad with a temperature of 31°C. In animals inoculated at the flanks with a temperature of 37°C, multiplication was recorded only when the dosage of *M. leprae* was high and there was no dissemination of the organism in any of them. The temperature at the site of entry and the dose of infecting *M. leprae* may play an important role in the development of leprosy in susceptible individuals exposed to *M. leprae.*

RESUMEN

Se investigó el efecto de la dosis de *Mycobacterium leprae* y el papel del microambiente en el sitio de inoculación, en la producción de lesiones de la lepra en los ratones T900r inmunológicamente suprimidos y altamente susceptibles. Se inocularon varias dosis de *M. leprae* (10^7, 10^6, 10^5 y 10^4) tanto en los flancos como en las almohadillas plantares de dos grupos diferentes de ratones. Los sitios de inoculación fueron muestreados para su examen histopatológico y para la cuenta de bacilos al final de 6, 8 y 12 meses de infección. Los bacilos inoculados en la almohadilla plantar (con una temper-

atura de 30°C) se multiplicaron a todas las dosis probadas en el sitio de inoculación y se diseminaron a otras partes del cuerpo. En contraste, en los animales inoculados en los flancos (con una temperatura de 37°C), la multiplicación ocurrió solo con las dosis altas de bacilos y no hubo diseminación de los mismos a ninguna concentración probada. Se concluye que la temperatura en el sitio de entrada y la dosis infectante de *M. leprae,* pueden jugar un papel importante en el desarrollo de la lepra en los individuos susceptibles, expuestos al bacilo.

RÉSUMÉ

Le rôle, pour l'obtention de lésions lépreuses, de la dose d'*inoculum* ansi que de l'environnement au site d'inoculation de *Mycobacterium leprae,* fut examiné en utilisant des souris hautement susceptibles, immunologiquement supprimées de souche T900r. Des doses de 10^7, 10^6, 10^5 et 10^4 furent inoculées, soit dans les flancs, soit dans la plante des pieds de deux différents groupes de souris. Des biopsies des sites d'inoculation furent réalisées pour évaluer les lésions histologiques et pour compter les bactéries après 6, 8 et 12 mois. *M. leprae* se sont multipliées localement et se sont disséminées à d'autres parties du corps à toutes les concentrations chez les souris infectées dans la plante des pieds, site à une température de 31°C. Chez les souris inoculées aux flancs à une température de 37°C, une multiplication ne fut enregistrée que lorsque les doses de *M. leprae* étaient élevées et aucune évidence de dissémination ne fut détectée dans ce groupe. La température au site d'entrée et la dose de *M. leprae* infectieuse pourraient jouer un rôle important dans le développement de la lèpre chez les individus sensibles exposés à *M. leprae.*

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