ATTEMPTS AT GROWTH OF *MYCOBACTERIUM LEPRAE* IN FOOTPADS OF MICE AND GUINEA-PIGS[1]

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INTRODUCTION

In 1960 Shepard ([12]) reported that inoculation of *Mycobacterium leprae* obtained from human patients into footpads of mice resulted in numerical increases of the acid-fast bacteria within the microscopic-sized granulomas they had produced. Subsequently, he confirmed and extended these findings ([13, 14]). Passage of the organisms through new groups of mice likewise resulted in bacterial multiplication. The identity of these acid-fast rods with *M. leprae* seems strongly supported by their failure to grow on artificial culture media or in cell cultures suitable for the propagation of most other mycobacteria. In a later publication Shepard and Chang ([15]) reported suppression of multiplication of *M. leprae* in footpads of mice treated with certain drugs, including 4,4′-diaminodiphenyl sulfone (DDS).

Shepard's findings have not yet been corroborated or extended to animal species other than mice.[2] The present communication is concerned with attempts in this laboratory to grow *M. leprae* in the footpads of mice and guinea-pigs. Some of the animals in these experiments were treated with cortisone acetate, prednisone, and Benadryl, as part of a more extensive investigation of the effect of drugs on native host resistance against infection with *M. leprae* and other mycobacteria.

MATERIALS AND METHODS

1. *M. leprae.*—A suspension of *M. leprae* was prepared from three-months-old subcutaneous embryomas of C_3H mice induced according to the method described by Robertsen ([11]). The excised embryomas were minced with scissors. They were then ground in a mortar with sterile sand with addition of Hanks' balanced salt solution (BSS). The resulting tissue-bacterial suspension was centrifuged at 5°C for 10 minutes at 1,000 r.p.m. The supernatant, diluted with BSS to contain the desired bacterial concentration, was used as inoculum.

2. *Bacterial enumeration.*—A volume of 0.2 cc. of the bacterial suspension was transferred to a screw-capped tube, and underlayered with 0.02 cc. of chloroform. The suspension then was alternately drawn up and expelled with a capillary pipette for at least one minute. Next sufficient 0.5 per cent phenol water containing 1 per cent

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[2]Since the manuscript was sent to the publisher the author learned that Shepard's findings have been confirmed by P. G. Janssens and S. R. Pattyn (presented at the VIIIth International Congress of Leprology, Rio de Janeiro, September 1963) and by R. J. W. Rees (personal communication). The author's later experiments with inocula obtained from nasal washings of untreated patients with lepromatous leprosy show appearance of increasing numbers and sizes of packets of acid-fast bacilli in the mouse footpads. The inocula did not contain culturable mycobacteria. These experiments are still in progress.

blood serum was added to bring the volume to 10 cc. The tube then was shaken vigorously and allowed to stand until the foam had disappeared.

To count the bacteria the suspension was spotted out on glass slides with a loop delivering a known volume. After drying in air the preparations were fixed with heat and the underside of the glass slide circled with a diamond pencil to locate the spots. The preparations were exposed for 5 minutes to formaldehyde vapor in a Coplin jar. They were then stained for 20 minutes with carbol-fuchsin at room temperature, decolorized with acid and counterstained with methylene blue in the usual manner.

To make the calculation, the number of oil-immersion fields along a diameter of the circular spot (d) and the average number of acid-fast bacilli per oil-immersion field ($\bar{n}$) were determined. The average number of bacilli per field multiplied by the square of the number of fields per diameter of the spot equals the number of bacteria (N) in the volume of the spot, which is known: ($N = \bar{n} \times d^2$).

3. *Animals used.*—(a) Mice: Adults of either sex of C_3H and C_{57} black strains were used. (b) Guinea-pigs: Adult albino guinea-pigs of either sex were used.

4. *Drugs used.*—(a) Cortisone: This drug was given to guinea-pigs only. They received intramuscular injections of 5 mgm. 3 times as week until they were sacrificed. (b) Prednisone: This drug was given to mice only. They were injected subcutaneously with 0.1 cc. of a saline suspension containing 0.05 mgm. of prednisone. This drug was administered 5 times a week until termination of the experiment. (c) Benadryl: This drug was administered to both guinea-pigs and mice by subcutaneous injection 5 times a week until the animals were sacrificed. Guinea-pigs received 8 mgm. and mice 0.08 mgm. of the drug per injection.

5. *Footpad injections.*—(a) Mice: Thirty mice of each of the two strains received a subcutaneous injection of 0.03 cc. of a suspension containing 140,000 leprosy bacilli into the pad of the left hind foot. The animals were injected in the pad of the right hind foot with an equal number of leprosy bacilli that had been killed by boiling the suspension for 15 minutes. (b) Guinea-pigs: Twenty-five guinea-pigs received in the pad of the left hind foot 0.1 cc. of a suspension containing 1,000,000 leprosy bacilli. An additional five animals received an equal number of heat-killed bacilli in the pad of the right hind foot.

6. *Drug treatment.*—(a) Mice: The mice of each strain were assigned to one of three groups of ten animals each. Group 1 received prednisone, Group 2 was treated with Benadryl, and the remaining group received injections of normal saline. (b) Guinea-pigs: Ten of the guinea-pigs injected with living *M. leprae* received Benadryl, ten cortisone and five normal saline. The five animals that had been inoculated with killed bacteria were also treated with normal saline.

7. *Examination of footpads.*—(a) Mice: The feet of mice that had died spontaneously or had been sacrificed at the times indicated in Tables 1 and 2 were removed and fixed in 10 per cent formaldehyde for varying lengths of time. Decalcification was carried out at room temperature in a solution of 5 per cent formic acid in 70 per cent ethanol as described by Shepard ([13]). Sections were cut 6 to 8 μ thick, and stained by acid-fast and hematoxylin-eosin stains. (b) Guinea-pigs: The footpad was removed with scissors and fixed in 10 per cent formalin. Sections 6 to 8 μ thick were stained with acid-fast and with hematoxylin-eosin stains. In the experiments with guinea-pigs Benadryl and saline injections were discontinued 180 days after infection of the animals. Cortisone treatment, however, was continued.

RESULTS

1. *Experiments with mice.*—The results of the experiments with mice have been summarized in Table 1 for C_3H mice and in Table 2 for C_{57} mice. Eight of the 30 C_3H mice had died by the 27th day fol-

TABLE 1.—*Results of footpad inoculation of* C_3H *mice with* M. leprae.

Number of mice	Treatment			Fate	Days after inoculation	Results
	Benadryl	Prednisone	Saline			
5	+			Died	4	No sections made
2	+			Died	11	AF[a] in one left footpad
1		+		Died	27	No AF
1	+			Sacrificed	107	No AF
3		+		Sacrificed	107	AF in 2 left footpads
3			+	Sacrificed	107	AF in one left footpad
2	+			Sacrificed	121	No AF
2		+		Sacrificed	121	No AF
2			+	Sacrificed	121	No AF
3		+		Sacrificed	220	No AF
2			+	Sacrificed	220	No AF
2		+		Sacrificed	310	Acid-fast debris
2			+	Sacrificed	310	No AF

[a]Acid-fast rods.

lowing footpad inoculation. Only in the left footpad of one of these animals was it possible to discover inoculated bacteria and these were in small number. A few acid-fast bacilli were seen in the left footpads of three of the seven mice sacrificed 107 days after infection. Two of the four mice sacrificed on the 310th day of the experiment had small amounts of acid-fast debris in their footpads. There was evidence neither of bacterial multiplication nor of granulomatous lesions as described by Shepard ([14]).

Four of the C_{57} mice had died by the 47th day following footpad inoculation. No acid-fast bacilli were found in the footpads of three of these animals, which had died on the 26th, 37th, and 43rd day, respectively. No sections were made from the footpads of the animal that died on the fourth day. Evidence of bacterial multiplication was lacking and there was no evidence of any tissue change that could be attributed to the presence of mycobacteria.

2. *Experiments with guinea-pigs.*—Data on the 30 guinea-pigs are summarized in Table 3. Acid-fast bacilli were seen in the footpad of only one of these animals. This particular guinea-pig had died four days after footpad inoculation. Acid-fast debris was seen in the footpads of four animals. One of these had died at 118 days and the three remaining guinea-pigs had been sacrificed 270 days after footpad inoculation. There were no intact bacteria in the footpad tissues of any of these animals. Here, as with the mice, there was no histologic evidence of any tissue change attributable to the presence of mycobacteria.

TABLE 2.—*Results of footpad inoculation of C_{57} mice with* M. leprae

Number of mice	Treatment			Fate	Days after inoculation	Results
	Benadryl	Prednisone	Saline			
1	+			Died	4	No sections made
1	+			Died	26	No AF[a]
1	+			Died	37	No AF
1	+			Died	43	No AF
3	+			Sacrificed	107	No AF
3		+		Sacrificed	107	No AF
3			+	Sacrificed	107	No AF
2	+			Sacrificed	121	No AF
2		+		Sacrificed	121	No AF
2			+	Sacrificed	121	No AF
3		+		Sacrificed	220	No AF
3			+	Sacrificed	220	No AF
1	+			Sacrificed	310	A few acid-fast granules
2		+		Sacrificed	310	No AF
2			+	Sacrificed	310	No AF

[a]Acid-fast rods.

DISCUSSION

Direct study of experimental leprosy depends on the availability of a susceptible laboratory animal that responds regularly and in a predictable fashion to infection with *M. leprae*. The generally negative or nonreproducible results of the many attempts to infect various species of animals (1) indicate that the growth requirements for this organism are not easily met within the tissue of experimental hosts. In this connection Feldman (2) has expressed the opinion that the skin might contain certain constituents essential for the initial multiplication of *M. leprae*. The existence of such substance in the skin, however, is entirely speculative. There are other reasons, however, why transmission experiments should include the intradermal route. As pointed out by Binford (1) the manifestations of leprosy in the human being suggest that the optimum growth temperature of *M. leprae* might be below that of internal organs of mammalian species. At least two other pathogenic mammalian mycobacteria fail to grow at temperatures as high as 37°C, viz., *M. ulcerans* and *M. balnei*. Each organism grows at temperatures of 30-33°C in artificial culture media. Using an electric "thermistor" thermometer, Binford (1) measured the temperatures at various body sites of the golden hamster (*Cricetus auratus*) at 16.1°C and 41.7°C.

It is interesting to note that not only intra-abdominal temperatures but also temperatures in the skin of the back of the hamster fell beyond the maximum growth temperature of *M. ulcerans* and *M. balnei*. Scrotum and testicle temperatures would seem suitable for the propa-

gation of bacteria with like temperature requirements under ambient temperature conditions that can be maintained in airconditioned animal quarters. The temperature in the skin of the earlobe of the Syrian hamster, on the other hand, was found to be surprisingly low.

Perhaps some such animal as the brown bat, *Myotis lucifigus*, might be used to advantage in leprosy transmission experiments, because this species maintains a body temperature close to the ambient temperature. This would facilitate a systematic investigation of the effect of temperature on infectivity.

Several factors might account for the failure of the present experiments to corroborate Shepard's findings ([12, 13, 14]). Among these is the fact that the temperature in the animal quarters always exceeded 18.9°C and occasionally reached 32°C. This is in sharp contrast to the conditions under which Shepard conducted his experiments. Another factor that might account for the observed discrepancy was the nature of the inoculated bacteria. Shepard prepared his inocula from

TABLE 3.—*Results of footpad inoculation of guinea-pigs with* M. leprae.

Number of guinea-pigs	Treatment			Fate	Days after inoculation	Results
	Benadryl	Cortisone	Saline			
1		+		Died	1	No AF[a]
1	+			Died	4	AF
1		+		Died	34	No AF
1	+			Sacrificed	56	No AF
1			+	Sacrificed	56	No AF
1			+	Died	62	No AF
1			+	Died	72	No AF
1		+		Died	81	No AF
1	+			Sacrificed	83	No AF
1	+			Died	118	Some acid-fast debris
1	+			Died	123	Too deteriorated for histologic examination
3		+		Sacrificed	195	No AF
1		+		Died	198	No AF
3		+		Died	216	No AF
1		+		Sacrificed	270	No AF
6	+			Sacrificed	270	Some acid-fast debris in 2 animals
5			+	Sacrificed	270	Acid-fast debris in 1 animal

[a]Acid-fast rods.

nasal washings and biopsy specimens of human patients, as well as from the mouse footpads of his own successful experiments.

As stated before, the bacteria in the present experiments were isolated from mouse embryomas. These contained a high proportion of granular bacteria. Recently, Rees and Valentine [10] have presented evidence strongly supporting the assumption that irregularly staining leprosy bacilli might be nonviable.

The mice used most extensively by Shepard were of the CFW strain. He obtained positive results also with C_{57} black mice [13]. The latter strain was used in the present experiments in addition to C_3H mice. Apparently Shepard found CFW mice and C_{57} black mice equally susceptible to footpad infection with *M. leprae*. Whether or not C_3H mice are as susceptible to footpad infection with *M. leprae* as CFW and C_{57} mice is not known at the present time. In experiments involving intraperitoneal infection of C_3H and C_{57} mice with *M. lepraemurium* evidence was obtained in this laboratory indicating a greater degree of susceptibility of C_{57} mice to infection with this organism [4]. Shepard [13] also made footpad inoculations with *M. leprae* in Syrian and Chinese hamsters and Mongolian gerbilles. He stated that microscopic granulomas developed in the footpads of these animals. He did not, however, present evidence of bacterial multiplication accompanying their development.

It is conceded that simultaneous injection of heat-killed *M. leprae* in one footpad of mice that received injections of *M. leprae* that had not been heat-killed, in the other footpad, in the present experiments, can be seriously criticized on the ground that such a practice might stimulate acquired resistance.

Guinea-pigs were inoculated in the present study because they have much larger footpads than small rodents and, therefore, might supply greater numbers of *M. leprae* than can be harvested from the latter kind of animals. That the number of bacteria obtainable from a mouse footpad is far too small to permit metabolic studies of *M. leprae* or immunologic studies of leprosy can be predicted from the following considerations. According to Shepard [14] one mouse footpad yields approximately 10^7 *M. leprae*. There are an estimated 300 to 500 million bacteria in one milligram of bacterial wet weight. Therefore, one would need at least 30-50 mouse footpads to obtain a single milligram of wet bacterial mass. Respiratory studies with bacterial suspension in the Warburg apparatus, employing flasks of about 15 cc. capacity, require somewhat less than 100 mgm. bacterial wet weight to obtain a reasonable rate of oxygen uptake [16]. Hence, one would need the bacteria from 3,000-5,000 mouse footpads to conduct measurements with a single substrate in a single flask. This, of course, is not practicable. Assuming a bacterial moisture content of 80 per cent and a protein content of 70 per cent of the dry mass, it would

take all the bacteria in from 210 to 350 mouse footpads to supply a single milligram of protein antigen of *M. leprae.*

This is a prohibitively expensive method of producing sufficient antigen for extensive immunologic investigations. In addition, it would be a practical impossibilty to recover the calculated amounts of protein from the bacteria. At best it might be possible to provide sufficient protein antigen for an investigation of the incidence of hypersensitivity to *M. leprae* protein in a limited population. It might be possible to elicit positive skin reactions in individuals infected with *M. leprae* with amounts of antigen equivalent to those in the second strength PPD test dose. If this were so, then 1 mgm. of *M. leprae* protein would provide 200 skin test doses.

In the present exeriments dealing with footpad inoculation with *M. leprae* the antihistamine Benadryl and corticosteroids were used to test the assumption that they might enhance the infectious process and promote spread of the infection beyond the site of inoculation. Attention should be drawn to the fact that neither Robertsen [11] nor Shepard [12, 13, 14] obtained any evidence that *M. leprae* had invaded adjacent or remote sites. As can be seen from the results in Tables 1 to 3, none of the drugs had any influence on the uniformly negative findings. In view of what has been said before concerning the temperature in the animal quarters and the quality of the inoculum, it seems conceivable that any enhancing drug action might have remained concealed. Nevertheless, it has been observed in extensive trials [4] that Benadryl failed to enhance multiplication of *M. leprae* in mouse embryomas and to promote spread of the microorganisms beyond the limits of the tumor.

Treatment of embryoma-bearing mice with sodium iodide also was ineffective in this respect [4]. Attempts to infect cortisone-treated [4] and Benadryl-treated guinea-pigs [5] and mice [4] with *M. leprae* failed to give any evidence of infection, even when the bacteria were introduced directly into macrophages of provoked peritoneal exudates. Injection of *M. lepraemurium* (Hawaiian strain) into macrophage-containing peritoneal exudates of guinea-pigs treated with Benadryl or pyrilamine maleate did not alter the usual course of the infection in this refractory species [5], contrary to previous claims by Kato [3]. According to Kato, guinea-pig macrophages seem to be endowed with an uncanny capability for rapidly disintegrating phagocytized *M. lepraemurium.* Treatment of the infected animals with pyrilamine maleate was said to deprive the macrophages of this ability. Experiments in this laboratory [5] failed to provide evidence of such intracellular destruction *in vivo* and *in vitro.* There is, on the other hand, considerable evidence that cortisone interferes with the capability of macrophages to inhibit intracellular multiplication of tubercle bacilli [6, 7]. Interestingly, this property seems to be shared by propyl thio-

uracil ([8]). There is no indication that cortisone depresses native resistance against *M. leprae* ([1, 4]), and its effect on *M. lepraemurium* infection of mice seems rather complex ([9]). Attempts at footpad infection of mice with *M. leprae* will be repeated in this laboratory under more propitious conditions in the animal quarters and with inocula prepared from the nasal washings and skin biopsies of leprous patients not previously treated with sulfones.

SUMMARY

1. Attempts have been made to propagate *M. leprae* in the footpads of mice and guinea-pigs.

2. To increase the likelihood of success, groups of mice were treated, following footpad inoculation of *M. leprae*, with Benadryl and prednisone. For the same reason, some guinea-pigs were treated with cortisone and with Benadryl.

3. The results of the footpad injections of *M. leprae* were entirely negative in both drug-treated and nontreated animals.

4. Factors that might have contributed to the failure to grow *M. leprae* in the footpads of mice and guinea-pigs have been discussed.

5. The results of some experiments in this laboratory which had the general aim of decreasing native resistance to infection with *M. leprae* and *M. lepraemurium* have been discussed.

RESUMEN

1. Se han hecho ensayos para propagar el *M. leprae* en las plantas del pie de ratones y cobayos.

2. Para aumentar la probabilidad del éxito, grupos de ratones despues de la inoculación del *M. leprae* en la planta del pie, fueron tratados con Benadril y prednisona. Por la misma razón, algunos cobayos fueron tratados con cortisona y Benadril.

3. Los resultados de las inyecciones del *M. leprae* fueron completamente negativos en ambos grupos de animales tratados o no con drogas.

4. Se discuten los factores que pueden haber contribuido al fracaso del crecimiento del *M. leprae* en las plantas del pie de ratones y cobayos.

5. Se discuten los resultados de algunos experimentos realizados en este laboratorio, los cuales han tenido como propósito disminuir la resistencia natural a la infección con *M. leprae* y *M. lepraemurium*.

RESUMÉ

1. Des essais ont été menés pour propager *M. leprae* dans la sole plantaire de souris et de cobayes.

2. Afin d'accroître les chances de succès, les groupes de souris ont été traités par le Benadryl et la prednisone, après inoculation de *M. leprae* dans la sole plantaire. Pour la même raison, quelques cobayes ont été traités avec de la cortisone et avec du Benadryl.

3. L'injection de *M. leprae* dans la sole plantaire a entraîné des résultats entièrement négatifs tant chez les animaux traités que chez les non-traités.

4. Les facteurs qui ont pu contribuer à l'échec de transmission de *M. leprae* dans la sole plantaire des souris et des cobayes ont été discutés.

5. Ont été discutés également les résultats de quelques expériences menées dans ce laboratoire dans le but de décroître la résistance innée à l'infection par *M. leprae* et *M. lepraemurium*.

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