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A Comparative Study of Mouse Foot Pad Inoculation of Skin Biopsy Specimens from Patients with Lepromatous Leprosy in San Francisco and Atlanta[1]

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During the past few years, several laboratories have reported failure to demonstrate consistently the multiplication of *Mycobacterium leprae* in the mouse foot pad ([2, 4]), as first described by Shepard ([6, 7]). Other laboratories, on the other hand, have reported successful reproduction of the technic ([1, 5]). When mouse foot pad inoculation was begun in San Francisco in 1967, a comparison of the results of mouse inoculation in San Francisco with those in Atlanta was planned. The results of this comparison may explain the failure of others to duplicate the technic and may also suggest a method of procedure to those investigators wishing to employ the technic for the first time.

METHODS

Skin biopsy specimens were obtained from patients with lepromatous leprosy attending the Leprosy Clinic of the U.S. Public Health Service Hospital, San Francisco. Specimens were most often divided; one portion was sent on wet ice by air to Atlanta for mouse inoculation, and the other portion was inoculated in San Francisco. Occasionally, two biopsies were performed on the same lesion, either at the same time, or within a few days. A portion of one biopsy was inoculated in San Francisco, and a portion of the second biopsy was inoculated in Atlanta. Inoculation in both laboratories was usually performed within 30 hours of biopsy. The technic employed in San Francisco is identical with that performed in Atlanta ([6, 7, 13]), with the exception that CFW mice from the National Communicable Disease Center's breeding colony are inoculated in Atlanta, whereas locally bred BALB/c mice are inoculated in San Francisco.

A variety of data are available to serve as a basis upon which to compare the results of mouse inoculation in the two laboratories. When a specimen is prepared for inoculation, the number of acid-fast bacteria (AFB) recovered from the specimen is recorded. The number of AFB inoculated into each right hind foot pad is $5x10^3$ when the concentration is sufficient, and inocula with a bacterial concentration greater than this are diluted appropriately. The volume of the inoculum is limited by the size of the mouse foot pad; therefore, when the concentration of AFB in the inoculum is insufficient to provide $5x10^3$ organisms per mouse, a smaller number of organisms is inoculated. Because the inoculum provides too few AFB to be seen in histopathologic sections of the mouse foot, multiplication of *M. leprae* in the mouse foot pad is monitored by means of monthly sacrifice of one mouse, beginning at three months after inoculation. The right hind foot is fixed in formalin, decalcified, and processed for histopathologic examination. The "incubation period" is defined as the number of months elapsed between inoculation and the first appearance of AFB in sections of the foot. Organisms are seen in the sections only after multiplication has occurred. When no AFB have been seen in any of the feet obtained by the time that 12 months have elapsed since inoculation, the specimen is considered to have contained no organisms capable of multiplication in the mouse foot

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pad—that is, inoculation of the specimen is concluded to have yielded a "negative" result. When a significant mycobacteria-containing lesion is noted, or when 30-50 cells in a section are seen to contain AFB, a harvest is performed of the pooled foot pad tissue from four mice. The number of organisms recovered at the time of harvest, calculated for each foot pad, and the number of days elapsed between inoculation and harvest permit the calculation of the crude doubling time, G, according to the formula:

$$G = \frac{\text{number of days}}{\log_2 \frac{\text{number of AFB recovered}}{\text{number of AFB inoculated}}}$$

When the incubation period is as long as nine to 12 months, inoculation of the specimen is concluded to have yielded a delayed result.

To permit monthly sacrifices for nine months and a harvest of the pooled foot pad tissue from four mice, it is necessary to inoculate enough mice. Twenty mice are routinely inoculated with each specimen.

Multiplication of *M. leprae* in the mouse foot pad follows a typical pattern. After a "lag phase" of approximately 60 days' duration, there is a period of logarithmic multiplication until a "plateau" somewhat greater than 10^6 AFB per foot pad is reached, usually between 150 and 180 days after inoculation.

RESULTS

The results of 23 specimens from 15 patients inoculated into mice in both laboratories are available for comparison. Identical results were obtained in 21 of the 23 specimens: 14 specimens yielded unequivocal evidence of multiplication of *M. leprae* in both laboratories; three specimens showed no evidence of bacterial multiplication in either laboratory; and four specimens yielded evidence of only delayed or irregular multiplication in both laboratories. Minor differences between the two laboratories were noted in the results of inoculation of only two specimens. In each case, evidence of delayed multiplication was found in San Francisco, whereas no evidence of multiplication was found in Atlanta.

TABLE 1. *Specimens yielding no evidence of multiplication of* M. leprae.

Specimen[a] No.	AFB recovered	AFB inoculated	Incubation[b] period (months)
1			
ATL	6.8×10^6	5.0×10^3	>12
SF	7.5×10^6	5.0×10^3	>12
2			
ATL	1.5×10^5	5.7×10^2	>12
SF	3.4×10^5	9.1×10^2	>12
3			
ATL	1.1×10^5	4.7×10^2	>12
SF	3.6×10^5	1.3×10^3	>12

[a] In this and subsequent tables, "ATL" indicates the results obtained at the National Communicable Disease Center, Atlanta; "SF" indicates those obtained at the Public Health Service Hospital, San Francisco.

[b] "Incubation period" has been defined in the text.

Table 1 shows the data obtained from the three specimens which yielded no evidence of bacterial multiplication in either laboratory. Close similarity of the numbers of AFB recovered from the specimens is evident. In the case of such specimens, a harvest is not performed in Atlanta. Harvests were performed in San Francisco, but in no case were AFB seen.

The four specimens which yielded delayed results in both laboratories are described in detail in Table 2. For these specimens, also, the similarity of numbers of organisms recovered in each laboratory is shown. The crude doubling time was longer than 50 days in each instance. The harvests were frequently low. Apparently when the number of infective organisms inoculated per mouse is low, not all the mice are infected. If a noninfected mouse is sacrificed for sections, the incubation period will appear lengthened. Furthermore, if some of the mice taken in the pool of four for harvest are not infected, the average number of AFB harvested per mouse will be decreased. The first entry in the table (Specimen No. 1, ATL) was an instance where the only mouse found infected was the one

TABLE 2. *Specimens yielding evidence of delayed or irregular multiplication of* M. leprae.

Specimen No.	AFB recovered	AFB inoculated	Incubation period (months)	Harvest		G[a] (days)
				Days	Number of AFB	
1						
ATL	4.5×10^{7}	5.0×10^{3}	9	285	$<5.4\times10^{4}$	>83.0
SF	4.0×10^{7}	5.0×10^{3}	12	379	6.2×10^{5}	54.9
2						
ATL	6.3×10^{6}	5.0×10^{3}	12	418	4.7×10^{5}	63.8
SF	3.0×10^{6}	5.0×10^{3}	9	304	2.2×10^{5}	55.7
3						
ATL	7.9×10^{5}	5.0×10^{3}	11	365	7.9×10^{4}	91.7
SF	7.2×10^{6}	5.0×10^{3}	11	365	2.8×10^{5}	62.9
4						
ATL	3.6×10^{5}	1.04×10^{3}	9	Not done		
SF	3.2×10^{5}	1.1×10^{3}	10	348	6.0×10^{3}	

[a] G = The crude doubling time, has been defined in the text.

taken for sections at nine months. Similar results have been seen with specimens taken from patients in the course of treatment just before their specimens became noninfectious (14). Some of the other entries (No. 3, ATL; No. 4, SF) had such low harvests that it appears unlikely that the harvest from even one of the mice in the pool was at normal plateau levels. Because these harvests were very late, it is possible that the number of bacilli was decreasing late in the plateau phase; such decreases have been observed in other experiments when apparently all of the mice had been successfully infected (10). Whatever the explanation, irregular results often result from inocula containing few infective organisms.

The two specimens which yielded discrepant results in the two laboratories, described in Table 3, demonstrate the similarity between a negative and a delayed result. If the delayed result may be explained by an inoculum that did not infect all of the mice, it is possible that a negative result may occur because none of the mice sacrificed to obtain foot pad tissue for histopathologic processing and examination will have received viable organisms in the inoculum, whereas others not so examined may

TABLE 3. *Specimens yielding no evidence of multiplication in Atlanta, and evidence of delayed multiplication in San Francisco.*

Specimen No.	AFB recovered	AFB inoculated	Incubation period (months)	Harvest		G (days)
				Days	Number of AFB	
1						
ATL	5.3×10^{6}	5.0×10^{3}	>12	Not done		
SF	7.8×10^{6}	5.0×10^{3}	11	379	4.7×10^{5}	58.1
2						
ATL	2.4×10^{7}	5.0×10^{3}	>12	Not done		
SF	3.8×10^{7}	5.0×10^{3}	10	386	1.0×10^{6}	50.5

have. If harvests from pooled tissues of four mice were performed routinely, the probability of recognizing multiplication of *M. leprae* might well be increased.

The results of the 14 specimens yielding evidence of the multiplication of *M. leprae* in both laboratories, summarized in Table 4, indicate the reliability with which this technic may be reproduced. That the results are not identical can be explained in

TABLE 4. *Specimens yielding evidence of multiplication of* M. leprae.

Specimen No.	AFB recovered	AFB inoculated	Incubation period (months)	Harvest		G (days)
				Days	Number of AFB	
1						
ATL	3.8×10^{7}	5.0×10^{3}	5	229	1.8×10^{5}	44.3
SF	1.5×10^{7}	5.0×10^{3}	4	258	8.0×10^{5}	35.2
2						
ATL	1.8×10^{5}	7.3×10^{2}	6	259	3.8×10^{5}	28.7
SF	5.5×10^{5}	2.2×10^{3}	7	281	3.8×10^{5}	36.7
3						
ATL	4.8×10^{7}	5.0×10^{3}	5	205	1.2×10^{6}	25.8
SF	8.4×10^{7}	5.0×10^{3}	4	250	2.2×10^{5}	45.8
4						
ATL	7.4×10^{7}	5.0×10^{3}	6	231	3.6×10^{6}	24.3
SF	6.1×10^{7}	5.0×10^{3}	—	214	1.5×10^{6}	25.9
5						
ATL	7.9×10^{7}	5.0×10^{3}	8	258	1.6×10^{6}	31.1
SF	6.4×10^{7}	5.1×10^{3}	6	231	1.3×10^{6}	28.8
6						
ATL	3.8×10^{6}	5.0×10^{3}	8	266	2.3×10^{5}	48.1
SF	1.8×10^{6}	5.1×10^{3}	6	275	8.9×10^{5}	37.0
7						
ATL	7.0×10^{6}	5.0×10^{3}	7	279	2.1×10^{6}	32.0
SF	1.8×10^{6}	4.8×10^{3}	5	231	1.2×10^{6}	29.1
8						
ATL	1.0×10^{7}	5.0×10^{3}	7	238	2.2×10^{6}	27.0
SF	5.2×10^{6}	5.0×10^{3}	6	267	1.7×10^{6}	31.9
9						
ATL	1.1×10^{7}	5.0×10^{3}	6	207	2.4×10^{6}	23.3
SF	2.8×10^{7}	5.0×10^{3}	6	210	7.6×10^{5}	29.0
10						
ATL	7.2×10^{5}	2.7×10^{3}	5	209	1.9×10^{6}	24.5
SF	7.7×10^{5}	2.6×10^{3}	6	238	7.8×10^{5}	32.6
11						
ATL	1.4×10^{7}	5.0×10^{3}	6	243	5.0×10^{5}	36.6
SF	9.2×10^{6}	5.0×10^{3}	5	265	7.0×10^{5}	37.2
12						
ATL	3.0×10^{7}	5.0×10^{3}	8	265	4.8×10^{5}	40.2
SF	2.9×10^{7}	5.0×10^{3}	6	274	8.2×10^{5}	37.3
13						
ATL	6.3×10^{6}	5.0×10^{3}	7	265	1.2×10^{6}	33.6
SF	4.7×10^{6}	5.0×10^{3}	5	237	1.1×10^{6}	30.7
14						
ATL	1.5×10^{8}	5.0×10^{3}	—	244	6.2×10^{5}	35.2
SF	9.9×10^{6}	5.0×10^{3}	—	174	7.9×10^{5}	23.8

part by inaccuracies in the timing of harvests; once multiplication is maximal, a delay in harvest will prolong the calculated crude generation time. Also, different strains of mice were employed in the two laboratories. Finally, it was necessary on a few occasions to biopsy different lesions. A recent study ([3]) has suggested that there may be important variation in the solid ratio (the proportion of brightly-and uniformly-staining organisms) and, therefore, of the proportion of viable organisms from lesion to lesion.

DISCUSSION

There are probably a number of reasons for the failure to achieve consistent evidence of multiplication of *M. leprae* after mouse foot pad inoculation. Among the possible causes are the use of poor inocula, such as might be obtained from treated patients ([14]) or from frozen material ([12]); the use of a mouse strain which does not permit good multiplication ([11]); and the failure to cool the animal quarters adequately ([9]). If one follows the published method ([6, 7]) without deviation, especially after a period of training in a laboratory in which the technic is established, there should be no difficulty. It is strongly recommended that the technic be undertaken only after such a period of training, and that comparability with a laboratory in which the technic has been established be measured, as has been done in this study.

SUMMARY

Portions of 23 skin biopsy specimens from patients with lepromatous leprosy have been inoculated into mouse foot pads both at the U.S. National Communicable Disease Center, Atlanta, Georgia, and at the U.S. Public Health Service Hospital, San Francisco, California. Identical results have been obtained for 21 of the 23 specimens: three yielded no evidence of multiplication of *M. leprae;* four yielded evidence of only delayed multiplication; and 14 demonstrated definite evidence of bacterial multiplication. Two specimens gave no evidence of multiplication in Atlanta, whereas they yielded evidence of delayed multiplication of *M. leprae* in San Francisco. Agreement between the two laboratories, while not perfect, was satisfactory.

If the originally described method is closely followed, especially after a period of training in a laboratory in which the technic is established, there should be no difficulty in duplicating the mouse foot pad technic.

RESUMEN

A 23 enfermos de lepra lepromatosa se le tomaron biopsias cutáneas, las cuales se dividieron en dos fragmentos. Con dichos fragmentos se hicieron inoculaciones en la almohadilla de la pata del ratón en el National Communicable Disease Center de Atlanta, Georgia y en el Public Health Service Hospital de San Francisco, California. Se obtuvieron resultados idénticos en 21 de las 23 muestras: 3 de ellas no evidenciaron multiplicación del *M. leprae*; 4 dieron evidencias de solamente multiplicación retardada y 14 demostraron evidencia definitiva de multiplicación bacteriana. Dos de las muestras no dieron evidencias de multiplicación es Atlanta, mientras que hubo evidencia de multiplicación retardada del *M. leprae* en San Francisco. La correlación entre los dos laboratorios, aunque no perfecta, fué satisfactoria.

Si se sigue fielmente el método descrito originalmente (6, 7), especialmente después de un período de entrenamiento en un laboratorio en el cual la técnica esté establecida, no deben presentarse dificultades para duplicar le técnica de la inoculación del *M. leprae* en la almohadilla de la pata del ratón.

RÉSUMÉ

Au National Communicable Disease Center d'Atlanta, en Géorgie, ainsi qu'à l'Hôspital du Public Health Service, de San Francisco, en Californie, des fragments de 23 échantillons de biopsies cutanées, provenant de malades atteints de lèpre lépromateuse, ont été inoculés dans le coussinet plantaire de la souris. Des résultats identiques ont été obtenus pour 21 de ces 23 échantillons. Trois d'entre eux n'ont montré aucun signe de multiplication de *M. leprae;* quatre ont témoigné seulement d'une multiplication retardée; quatorze ont montré des signes nets de multiplication bactérienne. Deux échantillons n'ont pas témoigné de multiplication à Atlanta, alors qu'ils montraient des signes de multiplication retardée de *M. leprae*

à San Francisco. La concordance des résultats entre les deux laboratoires, encore qu'elle ne soit pas parfaite, est considérée comme satisfaisante.

Lorsque l'on suit rigoureusement la méthode originalement décrite, particulièrement après une période de formation dans un laboratoire où cette technique est utilisée, il ne devrait pas se présenter de difficultés pour obtenir des résultats reproductibles avec la technique d'inoculation dans le coussinet plantaire de la souris.

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