MULTIPLICATION OF *MYCOBACTERIUM LEPRAE* IN THE FOOT-PAD OF THE MOUSE[1]

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The mouse foot-pad has been shown by Fenner ([3]) to be a favorable place for the multiplication of two of the mycobacterial species that cause human skin disease. The first is *Mycobacterium balnei* (probably equivalent to *M. marinum*), a relatively rapidly-growing photochromogenic species that causes "swimming-pool granuloma" reported from Sweden and the United States. The second is *M. ulcerans*, a very slow-growing species that causes a mycobacterial skin ulcer in tropical Australia and the Congo. The growth of both of these microorganisms on bacteriologic media is characterized by a temperature optimum of 31-33°C, little, if any, growth occurring at 37°C. When injected intravenously into the mouse, they multiply and produce disease especially in the hairless, peripheral portions (feet, tail, nose, etc.), in apparent manifestation of the temperature optimum below 37°C.

Success in the mouse foot-pad with these two cultivable mycobacterial agents of human skin disease suggested to me that leprosy bacilli might also grow in this environment. My first experiments were initiated in 1957 with *M. leprae* from the nasal washings of leprosy patients, and were later expanded to include such bacilli from skin biopsy specimens. Multiplication of the injected bacilli has occurred in most instances. The growth is very slow, and reaches its maximum (10^5 to 10^7 bacilli per foot-pad) in 5 to 10 months after an injection of 10^3 to 10^4 of bacilli.

An essential part of the work has been the use of a method for the accurate counting of the acid-fast bacilli, so that the inoculum and the harvest in each passage are known within close limits. The timing of the harvest has been set by results of the microscopic examination of stained sections cut from the decalcified feet of mice sacrificed at monthly intervals.

MATERIALS AND METHODS

The technique used for counting the acid-fast bacilli has been described ([7, 9, 10]). Several improvements in the procedure were made during the more than 3 years involved. The lower limit of detectability is usually about 4.5×10^2 acid-fast bacilli in the 0.03 cc. of inoculum; figures less than this amount were arrived at by the "complete method" ([9]).

Mice of the CFW strain, unless otherwise noted, were inoculated superficially into the right hind foot-pad with 0.03 cc. This volume balloons up the skin temporarily and

[1]The *Journal of Experimental Medicine* has kindly granted permission for the use of material from an earlier publication ([10]).

distributes itself throughout the length of the foot-pad. Usually 20 mice were inoculated in a group, and they were kept in a room at 70°F (21°C). One animal from each group was sacrificed each month, the foot was removed, a slit was cut in the dorsum, and the foot was fixed at least 3 days in buffered 10 per cent formalin. Decalcification was carried out with daily changes of 5 per cent formic acid in 70 per cent ethanol. About 20 days are required at room temperature, or 7 days at 37°C; higher temperatures affect nuclear staining. Several other decalcifying procedures said to be more rapid have been tried with unsatisfactory results. Sections were cut 6 to 8 μ thick, and stained with hematoxylin, azure and eosin (H-A-E), and by Fite's 1947 acid-fast procedure.

Harvests were performed by washing the foot with soap and water, rinsing with sterile water, drying with sterile gauze, and cutting off the foot-pad with scalpel and forceps. The tissue was then minced thoroughly with sharp scissors, placed in the cup of a Mickle disintegrator with about 20, 3 mm. glass beads, and 2 cc. of Hanks' balanced salt solution (BSS) was added. One minute of vibration with 5 mm. amplitude was used. Such treatment had been found not to lower the colony count of a suspension of *M. tuberculosis,* nor did it affect the viability of the more fragile *Pasteurella tularensis* [7]. The fluid from the Mickle cup was transferred to a tube, its volume made up to 2.5 cc. with BSS, and bovine albumin added to 0.1 per cent. Clumps of tissue were allowed to settle for 2 minutes, and the supernate was carefully removed with bulb and pipette and made up to 2 cc. with 0.1 per cent bovine albumin in BSS. Sometimes this step had to be repeated. The volumes were recorded at each operation and taken into account in the final calculation of the number of acid-fast bacilli harvested per foot.

Transfers of strains to new groups of mice were scheduled after examination of the stained sections showed the presence of a "significant" lesion, that being defined as one occupying at least one-quarter of a microscopic field with 12 × 45 magnification (12× ocular, 45× objective) (see later). For transfers, the current practice is to sacrifice four mice for the harvest, and this is usually accomplished within 6 weeks after the time the animal showing the "significant" lesion in the section was sacrificed. Earlier practices varied, in general toward fewer mice sacrificed for the transfer and more delay in accomplishing it.

The preparation of the nasal washings has been described [9, 11]. The biopsy specimens were treated by the method described above for foot-pads, and about 0.1 gm. was processed. The site selected for obtaining the specimen was usually the back. The patient's skin was prepared by thorough swabbing with tincture of iodine, and the iodine was removed with alcohol swabs. The biopsy specimens were collected at the U. S. Public Health Service Hospital, Carville, La., and the nasal washings at Carville and at the Central Luzon Sanitarium at Tala, near Manila. Air transport with wet-ice refrigeration was employed.

Materials to be inoculated were kept in an ice bath. Infectious precautions were observed, and most of the operations were carried out in a biologic safety cabinet.

RESULTS

Histologic response.—Lesions in the mouse foot-pad have only irregularly progressed to a size visible to the naked eye, and it has therefore been necessary to rely on the microscopic response.

The distribution of the inoculum could be seen in sections of mice sacrificed the next day after inoculation, if large numbers of bacilli (more than 10^5) had been injected. Usually the bacilli were concentrated near the groups of vessels and nerves that overlie the muscle bundles, or they were located in the fascial separations in the muscles. In C57 mice they sometimes extended between the metatarsal bones to the

dorsum. The lesions that later developed conformed to this distribution and showed little evidence of spread.

If the inoculum contained less than 10^5 bacilli, there followed a distinct incubation period during which the sections were normal (Fig. 1). Sometimes the earliest lesions contained only a few cells with acid-fast bacilli.

Characteristically there was an increase in the size of the lesion and in the number of bacilli in the next few months. Often a granuloma was seen to occupy several 12 x 10 microscopic fields, but the larger granulomas were not associated with larger numbers of bacilli. Sometimes, especially in the younger lesions, there were many acid-fast bacilli but there was no discernible cellular infiltration in the H-A-E section. The infiltrate consisted of large round cells, that is, macrophages and epithelioid cells; lymphocytes were usually infrequent. The bacilli appeared to lie within cells in all cases, and except in very old lesions they were brightly stained. The nerves were observed to be involved only rarely.

The histologic appearances were the same in primary passages of biopsy and nasal washing material, and in further passages.

The microscopic appearance of the sections has been used as a signal of the time for transfer of a strain to a new group of mice. For

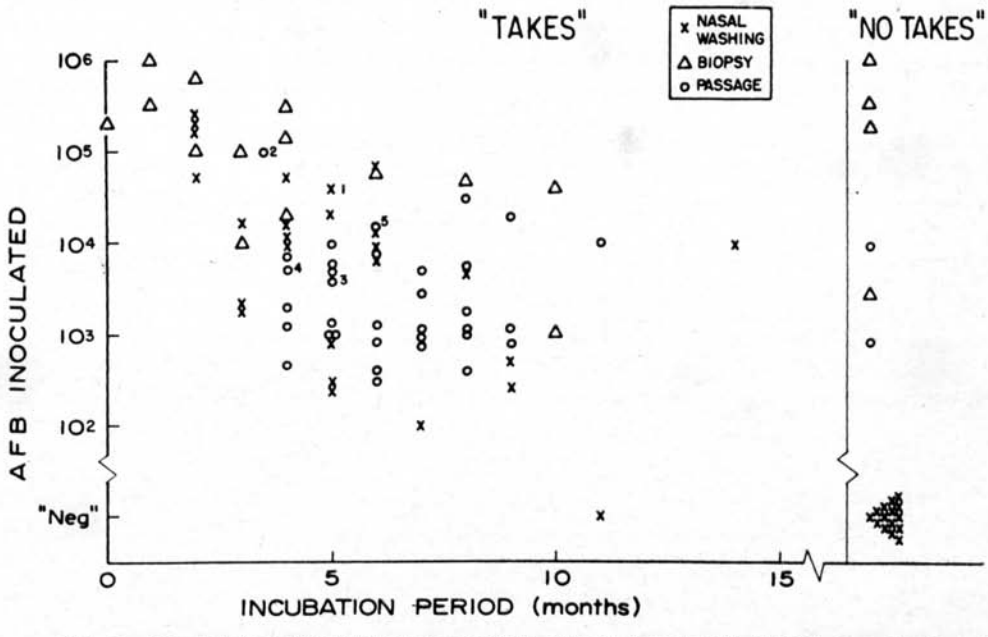

FIG. 1.—The relationship between the number of acid-fast bacilli (AFB) inoculated and the incubation period (time before development of a significant microscopic lesion). The results are given for "takes" (those producing significant lesions) and for "no takes" (significant lesions not produced). "Neg" refers to materials that did not contain enough AFB to be detected during the counting procedure, i.e., they contained less than 4.5×10^2 AFB in the 0.03 cc. inoculum. The numbers indicate the successive passages of the oldest strain, N2366.

this purpose a "significant" lesion is defined—as has been said—as one filling at least one-fourth of a 12 x 45 microscopic field with a typical infiltrate containing brightly-stained acid-fast bacilli, or an area the same size containing the bacilli without infiltrate.

The histologic response following the injection of leprosy bacilli is distinctly different from that which follows the injection of *M. ulcerans, M. balnei,* and the bacillus from the hamster isolates described by Binford (1), each of which is distinctive in its turn. Lack of necrosis is the essential distinguishing feature of the lesions that result from leprosy bacilli. Fenner (3) has found that neither the human nor the avian tubercle bacillus multiplies in the mouse foot-pad.

Relationship between the number of acid-fast bacilli in the inoculum and the incubation period.—This is given in Fig. 1, in which all of the results obtained to date are plotted. The greater the inoculum the shorter was the incubation period, in accord with the usual picture in infectious diseases. Inocula of $10^{2.5}$ to $10^{4.5}$ were followed by an incubation period of 3 to 9 months, with two exceptions.

Relationship between the number of acid-fast bacilli in the inoculum and the number of bacilli harvested at time of passage. This most essential correlation is shown in Fig. 2, in which again all of the data obtained have been plotted. Points falling above the heavy line marked 1X represent decrease in numbers of organisms; those below that line represent an increase.

The number of bacilli harvested does not particularly depend on the number inoculated. The 3 unfavorable points marked D are associated with an unusually delayed harvest, that is, the harvest was delayed 4 to 8 months after the first "significant" lesion had developed. These three occasions excepted, inocula of $10^{2.0}$ to $10^{4.0}$ have been followed by distinct increases in nearly every instance. Figs. 1 and 2 show the results of inoculating too large a dose, such as may result from the inoculation of skin biopsy material, in which very large numbers of bacilli may be found. Large inocula ($10^{5.0}$ to $10^{6.0}$) were followed by early appearance of granulomas in the absence of distinct bacillary increase. In the early stages of this work large numbers were used whenever possible. It was not until the last year that this relationship was realized. The present practice is to dilute the inoculum when necessary to contain 5×10^3 bacilli.

On passage, the strains have not altered their behavior as regards the relationship between inoculum and harvest.

Consistency with which "takes" are produced.—Information on this point is given in Table 1. A "take" is defined as the production of a "significant" lesion. (*a*) Following the injection of nasal washings containing detectable acid-fast bacilli, such lesions have been produced in 32 of 33 instances. The single failure is from a small inoculum (4.8×10^2) which has been followed for 14 months so far. (*b*) Following the

injection of nasal washings in which acid-fast bacilli were not found, a take has occurred in only 1 of 16 instances. The positive result appeared 11 months after the injection of a nasal washing from a patient whose biopsy-specimen organisms also produced a take. The patient's disease was diagnosed lepromatous on the basis of a biopsy. He had received irregular treatment several years before. It seems likely that leprosy bacilli were present in the nasal washings, but in numbers less than that needed for microscopic detection. (*c*) Following the injection of leprosy bacilli from skin biopsy specimens, takes occurred in 18 of 22 instances.

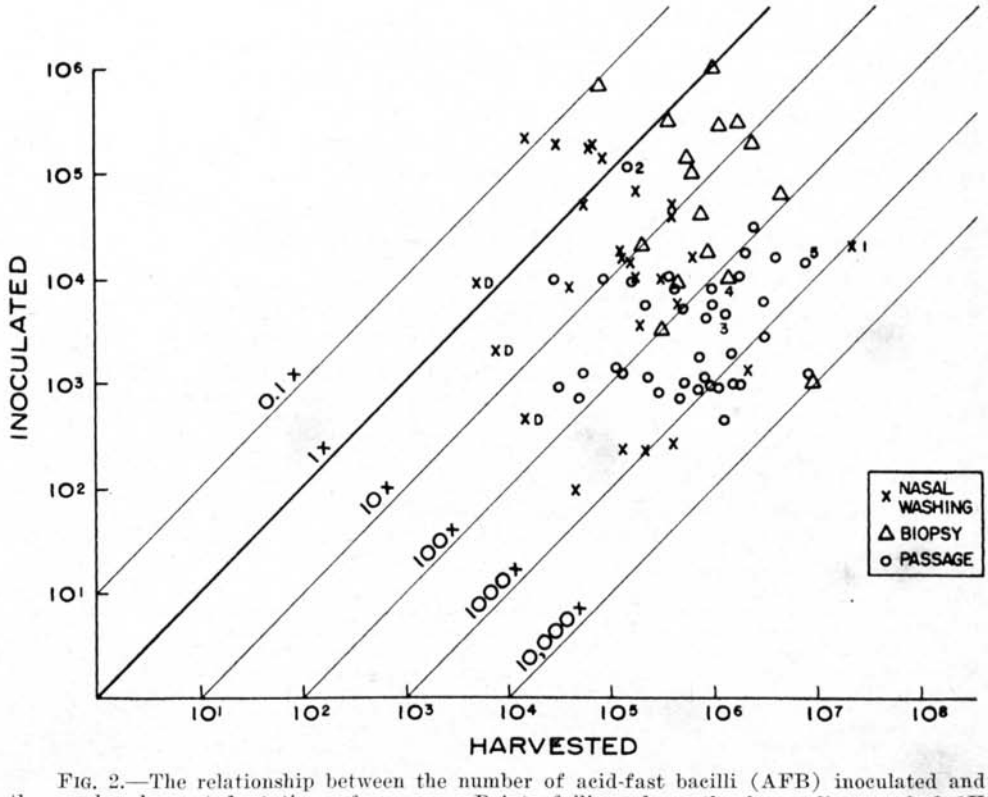

FIG. 2.—The relationship between the number of acid-fast bacilli (AFB) inoculated and the number harvested at time of passage. Points falling above the heavy line marked 1X represent decreases in number of bacilli; those below, increases. The three marked D were associated with unfavorably delayed harvests. The numbers refer to successive passages of the oldest strain, N2366.

TABLE 1.—*Consistency with which "takes" are produced according to source of inoculum and the numbers of acid-fast bacilli inoculated.*

Source of material	No. of acid-fast bacilli inoculated	No. of "takes" / No. of inoculations
Nasal washings	$<4.5 \times 10^2$	1/16
Nasal washings	9×10^1 to 2×10^5	32/33
Biopsy specimens	9×10^1 to 9×10^5	18/22
Passage material	4.5×10^2 to 1×10^5	49/51

The failures occurred early in the work, and their significance is hard to assess. (*d*) Following the injection of passage material containing acid-fast bacilli, takes developed in 49 of 51 cases. The two failures occurred early in the work, before the timing of harvest was understood.

The overall picture is thus one of high consistency, and it fits an interpretation that lesions are produced very frequently following the injection of leprosy bacilli, and not in their absence.

Results with passages.—Continued multiplication has occurred on passage in accord with the relationships of Fig. 2. With the oldest strain, N2366, five passages have been completed, with a total increase of 6×10^9-fold. Another nasal strain has completed four passages with a total increase of 8×10^6-fold. Seventeen strains have completed 3 passages, with total increases of 1×10^4- to 7×10^8-fold. Thirty-one strains have finished two passages; 7 of these are tissue strains from biopsy specimens.

There has been no discernible change on passage of the strains as regards appearance of the lesions, incubation period, or amount of harvest. The characteristic picture produced by the injection of leprosy bacilli from patients is still being produced on continued passage. It is hoped that this point can be established with more certainty, and it is planned to attempt to carry about 24 nasal strains and 12 tissue strains through four or five passages.

Rate of multiplication.—The generation time has been calculated from the number of acid-fast bacilli inoculated, the number harvested, and the time from inoculation to harvest. The calculation itself does not take into account a possible lag phase or stationary phase. The effect of these two factors is proportionately less, however, with smaller inocula. The generation time is given in Fig. 3 according to the size of inoculum for the data obtained since September 1959, when several improvements were made in the counting techniques. With inocula in the range 10^3 to 10^4, generation times of 20 to 30 days were frequent.

Ability of the bacilli to withstand shipment.—Passage inoculations were accomplished within a few hours. Shipments of materials from Carville for primary inoculation were made under wet-ice refrigeration, and from the time of collection until the mice were inoculated about 24 hours usually elapsed. The Philippine specimens were delayed en route, and about 100 hours had passed before inoculation; they were, however, re-iced twice en route and were received well iced. Of the 8 Philippine specimens, 6 contained acid-fast bacilli and 2 were controls without bacilli. The mice receiving the positive specimens developed takes that were normal in histologic appearance and in their positions in Figs. 1 and 2. The two control specimens did not produce takes. Thus there was no evidence that the bacilli deteriorated en route. The length of time elapsed would allow air shipment under normal conditions from any part of the world near air routes, if re-icing en route could be arranged.

Intratesticular inoculations of CFW mice.—For a period of 12 months the mice inoculated into the foot-pad were also inoculated intratesticularly with about 0.01 cc. or about one-third of the foot-pad inoculum. A total of 16 nasal washings, 17 biopsy specimens, and 22 passage materials were inoculated. Thus 55 materials were compared in approximately 1,100 mice. Sections cut after the monthly sacrifices failed to reveal any superiority for the testicular route of inoculation, since acid-fast bacilli did not appear earlier in that organ or develop in larger numbers.

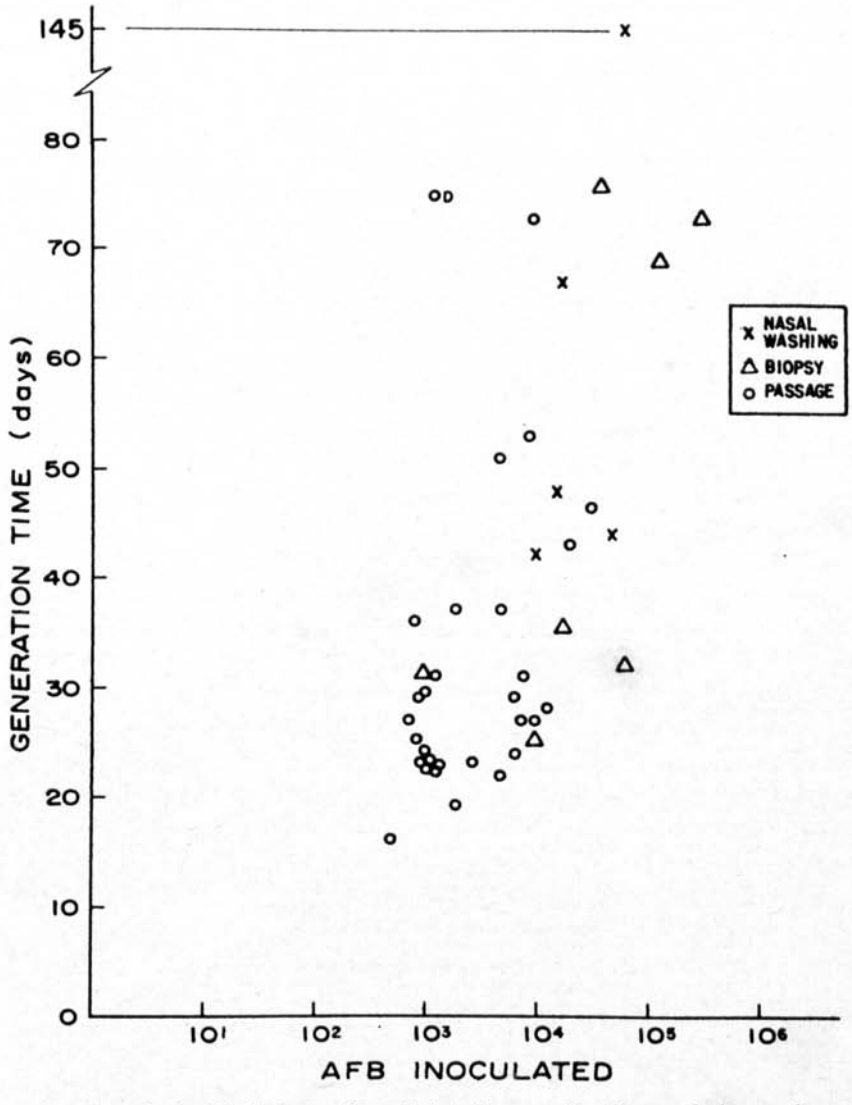

FIG. 3.—The relationship between the calculated generation time and the number of acid-fast bacilli (AFB) inoculated. The point marked D refers to an unfavorably delayed harvest.

Harvests of the acid-fast bacilli from the testicle were compared with harvests from the foot-pads in 43 instances. In 8 cases the testicular harvests were superior (the testicular harvests were 14.2 to 0.8 times the foot harvests). In 12 cases the testicular harvests were ranged from 0.53 to 0.10 times the foot harvest, indicating that the amount of multiplication in the two sites had been about the same. In 11 instances the testicular harvests were distinctly lower (0.088 to 0.0037 times the foot harvest). In 12 other instances the testicular harvests were so low that no acid-fast bacilli were seen during the microscopic search. It seems clear that multiplication of leprosy bacilli in the mouse testicle did occur on occasion, but the results did not have the high degree of consistency that followed the foot-pad inoculations.

Inoculation of C57 mice.—In a study of the susceptibility of different strains of mice to experimental tuberculosis, Pierce *et al.* ([5]) found the C57 strain to be among the most susceptible. For some time in the present study C57 mice were inoculated in parallel with CFW mice. During this comparison 26 materials were inoculated, comprising 12 nasal washings, 12 biopsy specimens, and 2 mouse-passage materials; this represented a total of about 520 mice. Sections of the animals killed at monthly intervals showed no distinct differences between the two strains of mice, although there was some tendency for the cellular infiltration to appear earlier in the C57 groups. Comparisons of harvests were made in 5 groups (3 biopsy specimens and 2 nasal washings). The ratio of the harvest from the C57 to that in the CFW mice was <0.024, 0.033, 0.091, 0.27, and 2.0. Thus there was no evidence of superiority of the C57 line for the growth of the bacilli. Since the C57 line was extensively infected with Salmonella and offered no advantages, its use was discontinued.

Inoculation of Chatterjee mice.—K. R. Chatterjee ([2]) employed a line of hybrid black mice from a cross between domestic and laboratory white mice, and reported that leprosy bacilli would grow in this strain to produce massive infections. Mice of this line recently have been available in limited numbers at the Communicable Disease Center, and they have been inoculated in parallel with CFW mice. Results are available from 6 materials inoculated, representing 1 biopsy specimen, 2 nasal washings, and 3 passages. Studies of the foot-pad tissue sections taken at the monthly sacrifices have sometimes shown superiority for the Chattejee line in numbers of bacilli. Spread of the infection from the foot-pad has been sought but not found. The ratio of the harvests from the Chatterjee mice to those from the GFW mice has been 0.83, 0.87, 0.91, 3.0, 3.2, and 66.1. Thus in three instances harvests were about the same, twice they were somewhat superior, and once markedly superior. Studies with this line of mice are continuing.

Inoculation of hamsters.—Binford ([1]) reported that histiocytic granulomas containing large numbers of mycobacteria developed in the

testicles of Syrian hamsters inoculated with specimens from skin containing leprosy bacilli. He observed similar results with two different inocula. The incubation period was 1 to 2 years on first passage, but it shortened to 4 months in second passage. The mycobacteria from the infected testicles can be readily grown on artificial media ([1]), especially 7H9 medium ([12]).

After Binford's report appeared, many of the materials inoculated into mice in this study were also inoculated into hamsters. Techniques for the hamster inoculations varied somewhat, but in most cases the inoculum was 7 times as dilute as that given the mice, and 0.2 cc. was inoculated into each testicle and 0.03 cc. into the right hind foot-pad. Hamsters were sacrificed for sections at infrequent intervals spread out over the time indicated by Binford's results. In all, 574 hamsters were inoculated with 83 materials consisting of 32 nasal washings, 19 skin biopsy specimens, 20 mouse passages, and 12 hamster passages. The findings in the tissue sections are condensed in Table 2. When acid-fasts were seen they were usually few in number. With the exception of the results with materials originating from patient 2403 (discussed below), the number of acid-fast bacilli was distinctly less than that seen in the mice inoculated in parallel and sacrificed at the same time.

TABLE 2.—*Incidence of acid-fast bacilli in sections of hamsters inoculated with materials also inoculated into mouse foot-pads; positives/number of hamsters inoculated and examined.*[a]

Site	Time of observation (months)							
	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24
Testicle	1/16[a]	1/36	3/42	4/38	2/44	0/22	0/15	0/1
Foot	1/7	1/15	2/19	3/9	2/18	0/5	3/15	0/0

[a]In most of the "positives" only a few acid-fast bacilli were seen. The "positive-results" materials originating from Patient 2403 are not included.

Patient 2403 material: Some of the hamsters receiving material from Patient 2403 have responded very differently. Thirteen months after inoculation of the nasal washings, 1 of the hamsters developed ascites and an enlarged spleen containing many acid-fast bacilli. Intratesticular transfer of spleen suspension to more hamsters resulted in relatively rapid growth of the mycobacteria (generation time about one week), and the strain is now in its sixth passage. It has not been possible to cultivate acid-fast bacilli on the media used (see below) incubated at 33°C.

From a hamster inoculated with biopsy-tissue bacilli from the same patient, a "blind" passage was made with testicular material. In the second-passage animals histiocytic testicular granulomas containing large numbers of bacilli have been observed at 9 months and more extensively at 15 months. The histology resembled very closely that described by Binford ([1]). Other hamsters in this group have remained

normal, and from them it has not been possible to recover acid-fast bacilli for culture and transfer.

On the basis of present evidence it cannot be said whether these microorganisms are leprosy bacilli or not. Their noncultivability makes them interesting in any case. Isolation had been made in mice from this patient's skin specimen and nasal washings, and the 2 mouse isolates and the hamster isolate are being compared in mice and hamsters.

Inoculation of tissue cultures.—Many of the materials inoculated into mice were also inoculated into tissue cultures of HeLa cells in media containing bovine fetuin and human albumin in place of serum ([8]). This medium contains only two electrophoretically-pure proteins, and when it is used as "infection medium" for tubercle bacilli there is unusually rapid intracellular growth of these organisms. No growth of leprosy bacilli was observed even though the bacilli could be observed in the cells for 2 months, which was the approximate life of the cell cultures. Earlier work with other media and other cells was likewise negative ([6]); monkey kidney cultures were followed for 2 to 4 months, and human amnion cultures for periods up to 9 months.

Inoculation of bacteriologic media.—Most of the materials inoculated into mice and harvested from them have also been inoculated onto bacteriologic media. For the last 20 months all such materials have been inoculated onto Loewenstein-Jensen medium, 25 per cent blood agar, 7H9 medium with oleic acid-albumin supplement as agar and as broth. They have been incubated at 33°C and observed for at least 4 months. Only the expected variety of nonacid-fast organisms have been grown, and these only very infrequently from the biopsy tissue and passage preparations.

DISCUSSION

The evidence that mycobacterial isolates described are the organism that causes human leprosy is as follows:

(*a*) "Takes" in mice were observed in 50 of 55 instances following the injection of leprosy bacilli from patients. The acid-fast bacilli counted in the preparations of skin biopsy specimens were leprosy bacilli, almost by definition. The evidence that the acid-fast bacteria in nasal washings were leprosy bacilli has been presented ([9, 11]). Although the takes resulting from injection of large numbers of bacilli were not always associated with multiplication, those from small doses were (Fig. 2). This point corresponds to the requirement that the organism be isolated with high frequency from patients with the disease.

(*b*) Takes were not produced following the injection of 16 nasal washings not observed to contain leprosy bacilli, except in one instance. In that instance it seems likely that the inoculum contained leprosy bacilli in numbers less than that required for microscopic detectability, that is, less than 450 bacilli in the inoculum. The "negative" nasal

washings were obtained from patients with treated lepromatous leprosy, and from untreated tuberculoid and borderline leprosy. This is analogous with the requirement that the organism not be isolated from healthy persons who are not carriers.

(*c*) Quantitative studies showed a relationship between the dose of leprosy bacilli injected and the incubation period. This is a frequent finding in infectious diseases: the infectious agent, when introduced in small numbers, multiplies for a period at a characteristic rate before it is able to produce the characteristic disease response. The results obtained here are compatible with the notion that the bacilli multiply with a generation time usually of 20 to 30 days until they reach a level of $10^{5.0}$ to $10^{7.0}$, at which time they are observable in the tissue sections and frequently cause the characteristic infiltration of large round cells. Thus the result was dependent not only on the presence of leprosy bacilli but also on their number. Such a relationship minimizes the possibility that an incidental contaminant has played a role.

(*d*) Continued passage through mice has not altered the quantitative relationships or the histologic response. This serves to make more certain the conclusion that multiplication of the bacilli has occurred, and that the result does not depend upon carry-over of the original inoculum. It also argues against the possibility that a latent mycobacterial disease in mice has been set off as a result of the injections.

(*e*) In the previous paper ([11]) a relationship was described between the severity of the clinical illness and the number of acid-fast bacilli in the nasal washings; a certain degree of correlation between the number of bacilli in skin lesions and the form of clinical leprosy has long been accepted. Since the number of acid-fast bacilli in the inoculum governed the incubation period (Fig. 1), the counts of acid-fast bacilli serve to connect the clinical manifestations of the natural disease to humans to an important characteristic of the experimental disease in mice.

(*f*) The microscopic anatomy of the experimental disease is compatible with that of the human disease. There is a slow growth of mycobacteria sometimes to high concentrations, there is usually a granuloma of large round cells, and there is no necrosis. The organisms appear to be chiefly intracellular. The histologic response in the mouse foot-pad with this microorganism is distinct from that caused by *M. ulcerans* and *M. balnei.*

(*g*) It has not been possible to culture mycobacteria from the inocula producing takes in mice, or from the harvests from them. The media used were those on which most known mycobacteria grow readily. Many of the same inocula failed to grow when put in tissue cultures under conditions suitable for the intracellular growth of many mycobacteria. These results with bacteriologic media and tissue cultures serve to rule out a very large number of mycobacterial species, and they minimize

the possibility that incidental mycobacterial contaminants, either from the patient or from the mice, have played a part.

Review of the reports in the literature on the isolation of leprosy bacilli emphasizes the usual sources of error. First is a common one in the isolation of infectious agents: the isolate is not the cause of the infection, but is instead a contaminant on the skin, in the tissues, in the medium, or in the animals inoculated. Mycobacteria are frequent organisms in man's environment, and one may be certain of culturing incidental mycobacterial species if he cultures diligently. As regards the present isolates this source of error seems minimized, since if a contaminant is responsible it would need to have been present only in patients with leprosy bacilli, present in concentrations proportional to the number of leprosy bacilli, capable of growing very slowly in mouse foot-pads to produce granulomas without necrosis, and incapable of growth on bacteriologic media under the conditions employed. The interpretation that the isolates are *M. leprae* seems more economical of hypotheses, and preferable according to Occam's razor.[2]

The other principal source of confusion in attempts to isolate *M. leprae* has arisen from the tremendous numbers of bacilli sometimes present in the patient's tissues. Hanks ([4]) has reported this range up to 10^9 bacilli per cubic centimeter of tissue, and my own unpublished results confirm that estimate. These very durable organisms remain demonstrable for long periods after their removal from the patient and inoculation into tissue cultures, bacteriologic media, or experimental animals. Without multiplication, mycobacteria are able to produce granulomatous responses in animal tissues.

The results shown in Fig. 1 indicate that only 10^5 to 10^6 of leprosy bacilli are able to cause a granuloma in the mouse foot-pad. It is not difficult to prepare materials containing many times this amount, and if passage conditions do not provide much dilution, one could "pass" the granulomas into new animals several times. As a control of this situation some investigators have performed parallel inoculations with heat-killed leprosy bacilli. When the control is positive it is most informative, because it shows that the effect could have been produced without bacterial multiplication. When the control is negative it has less value, because killed bacteria are notoriously sensitive to enzymatic digestion. There does not appear to be a way of rendering the bacteria incapable of multiplication without also making them more susceptible to external enzymes.

It seems clear that, in the present work, persistence can be ruled out. The minimum number of leprosy bacilli required to produce a

[2]William of Occam, an English philosopher who lived from about 1300 to 1349, was influential in leading thought away from excessively elaborate clerical dogmas of his day. The principle referred to as his Razor states that a simpler hypothesis is logically preferable, on the basis that it is not sound to explain the discrepancies of one hypothesis by invoking still another.

lesion without further multiplication in mouse foot-pads is about 10^5, yet when smaller numbers were injected they were able to multiply to this level and cause lesions. On passage, the infection behaves characteristically according to the number of bacilli in the inoculum, and the oldest strain has now multiplied about 6 billion-fold.

The number of leprosy bacilli produced in the mouse foot-pad is in the range of 10^5 to 10^7, and this corresponds to approximately 10^6 to 10^8 bacilli per gram of tissue. Hanks (4), as has been said, found the number of leprosy bacilli per cubic centimeter of lepromatous nodules to range from 6×10^8 to 7×10^9. In unpublished work we have found the number of leprosy bacilli per gram of skin biopsy tissues of 20 untreated lepromatous patients at Carville to range from 4×10^6 to 2×10^9, with the median value about 1×10^8. The ranges of the numbers of leprosy bacilli per gram of infected mouse tissue thus overlapped that of human lepromatous tissue. The tissue of tuberculoid leprosy patients contains much fewer bacilli.

SUMMARY

1. Leprosy bacilli injected into the foot-pads of CFW mice multiplied locally when the inoculum was appropriately diluted. The incubation period depended on the number of bacilli inoculated, averaging 2 to 3 months with 10^5 to 10^6 bacilli, and 5 to 7 months with 10^2 to 10^3 bacilli. Following the incubation period, acid-fast bacilli were found in the sections, and 10^5 to 10^7 acid-fast bacilli could be harvested.

2. Such positive results have developed with reasonable consistency following the injection of leprosy bacilli from clinical materials, having appeared in 32 of 33 instances following the injection of bacilli from nasal washings, and in 18 of 22 instances after injection of bacilli from skin biopsy specimens. They have appeared in only 1 of 16 instances following the inoculation of patient's nasal washings in which bacilli had not been detected microscopically.

3. Passage into new groups of mice has also been consistent, having been successful in 49 of 51 instances. The experimental infection has not changed in passage and gives the same harvest of acid-fast bacilli, histologic response, and incubation period. With inocula containing 10^3 to 10^4 bacilli, the increase in each passage has been 50- to 1,000-fold. At the time of writing, the oldest strain has completed 5 passages with a total increase 6×10^9-fold. Seventeen strains have completed 3 passages, with total increases of 1×10^4- to 1×10^7-fold. Thirty-one strains have finished 2 passages.

4. The generation time of the bacilli appears to be usually 20 to 30 days when small inocula are used.

5. Cultures on bacteriologic media favorable for the growth of most mycobacteria have been negative.

6. Parallel foot-pad inoculations of C57 mice gave similar results. Parallel intratesticular inoculations of CFW and C57 mice resulted in distinct multiplication in many instances, but the result was less regular. Parallel experiments with mice originating from Chatterjee's hybrids have not given the massive infections reported by him.

7. Hamsters inoculated intratesticularly have not given the results observed by Binford. In one instance an unknown mycobacterial infection developed; it is transferable by passage, but it has not been possible to cultivate the organism on bacteriologic media.

RESUMEN

1. Los bacilos leprosos inyectados en los cojinetes de la planta del pie se multiplicaron localmente cuando el inóculo estaba diluído en forma adecuada. El período de incubación dependió de la cantidad de bacilos inoculados, promediando de 2 a 3 meses con 10^5 a 10^6 bacilos y de 5 a 7 meses con 10^2 a 10^3 bacilos. Después del período de incubación, se descubrieron bacilos ácidorresistentes en los cortes, pudiendo cosecharse de 10^5 a 10^7 de ellos.

2. Esos resultados positivos se han presentado con bastante constancia a continuación de la inyección de bacilos leprosos procedentes de piezas clínicas, habiéndose observado en 32 de 33 casos después de la inyección de bacilos derivados de lavados nasales y en 18 de 22 casos después de inyectar bacilos de ejemplares de biopsias cutáneas. No se observaron más que en 1 de 16 casos consecutivamente a la inoculación de lavados nasales del enfermo cuando no se habían descubierto microscópicamente bacilos en ellos.

3. Ha sido también constante el pase a nuevos grupos de ratones, habiéndose logrado en 49 de 51 casos. La infección experimental no se alteró con el pase y da la misma cosecha en bacilos ácidorresistentes, respuesta histológica y período de incubación. Con inóculos que contenían de 10^3 a 10^4 bacilos, el pase ha sido de 50 a 1000 veces mayor. A la fecha de este trabajo, la cepa más antigua ha completado 5 pases con un aumento total de 6×10^9. Diecisiete cepas han terminado 3 pases con aumentos totales de 1×10^4 a 1×10^7. Treinta y una cepas han terminado 2 pases.

4. El tiempo de generación de los bacilos parece ser habitualmente de 20 a 30 días cuando se usan pequeño inóculos.

5. Los cultivos en medios bacteriológicos favorables para la proliferación de la mayoría de las micobacterias resultaron negativos.

6. Las inoculaciones paralelas en el cojinete de la planta del pie de ratones C57 dieron resultados semejantes. Las inoculaciones intratesticulares paralelas en ratones CFW y C57 dieron por resultado una multiplicación distinguible en muchas ocasiones, pero el resultado fué menos regular. Los experimentos paralelos con ratones derivados de los híbridos de Chatterjee no han dado infecciones masivas descritas por dicho autor.

7. Los *Cricetus* inoculados intratesticularmente no han dado los resultados observados por Binford. En una ocasión se presentó una infección micobacteriana desconocida; es transferible por pases, pero no ha sido posible cultivar el microbio en medios bacteriológicos.

RESUMÉ

1. Des bacilles de la lépre injectés dans la plante des pattes de souris CFW se multiplient localement, lorsque l'inoculat a été dilué de maniére adéquate. La période d'incubation dépend du nombre de bacilles inoculés, et varie de deux à trois mois avec 10^5 à 10^6 bacilles, de 5 à 7 mois avec 10^2 à 10^3 bacilles. Lorsque la période d'incubation est écoulée, des bacilles acido-résistants ont été trouvés dans les coupes, et 10^5 à 10^6 acido-résistants ont pu être recueillis.

2. De tels résultats positifs ont été retrouvés de manière raisonnablement concordante aprés injection de bacilles de la lèpre provenant de matériel clinique. Ils sont en effet apparus 32 fois sur 33 á la suite d'injection de bacilles provennant de lavages des cavités nasales, et 18 sur 22 fois après injection de bacilles recueillis à partir d'échantillons de peau prélevés pour biopsie. Ils n'ont été enregistrés que dans un seul cas sur 16 par inoculation de produits de lavage nasal dans lesquels des bacilles n'avaient pu être décelés par l'examen microscopique.

3. Le passage sur de nouveaux groupes de souris, effectué avec succès dans 49 cas sur 51, donne également des résultats cohérents. L'infection expérimentale ne s'est pas modifiée par le passage, et fournit les mêmes données en ce qui concerne les bacilles acido-résistants recueillis, la réaction des tissus, et la période d'incubation. Avec des inoculats de 10^3 à 10^4 bacilles, l'accroissement entraîné à la suite de chaque passage se situe entre 50 et 1000 fois. Au moment de rédiger cette communication, la plus vieille souche a subi 5 passages, l'accroissement total étant de six milliards de fois. Dix-sept souches ont achevé trois passages, avec un accroissement total de 1×10^4 à 1×10^7 fois. Trente et une souches ont terminé 2 passages.

4. Le temps de reproduction des bacilles se situe généralement entre 20 et 30 jours lorsque l'on procéde avec de petits inoculats.

5. Les ensemencements ont été négatifs sur des milieux de culture favorables à la croissance de la plupart des mycobactéries.

6. Des inoculations analogues dans la plante des pattes de souris C57 ont donné des résultats similaires. Des inoculations intratesticulaires analogues chez des souris CFW et C57 ont été suivies de multiplication notable dans bien des cas, mais les résultats ont été moins réguliers. Des expériences menées parallélement chez des souris issues des hybrides de Chatterjee n'ont pas entraîné l'infection massive qu'il a rapportée.

7. Des inoculations intratesticulaires chez le hamster n'ont pas donné les résultats observés par Binford. Dans un cas, une infection mycobactérienne inconnue s'est développée; elle peut être transférée par passage, mais il n'a pas été possible de cultiver l'organisme en cause sur milieu de culture.

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