THE EXPERIMENTAL DISEASE THAT FOLLOWS THE INJECTION OF HUMAN LEPROSY BACILLI INTO FOOT-PADS OF MICE

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In the lepromatous form of human leprosy the nasal passages are commonly involved, and large numbers of acid-fast bacilli can be recovered in the nasal washings by methods that have been described (1). That the acid-fast bacilli obtained in this fashion are in fact *Mycobacterium leprae* is evidenced by a number of considerations (1), which include the close correlation of the number of acid-fast bacilli recovered with the typical clinical manifestations of the disease, the characteristic microscopic arrangement of the bacilli in globi and packets, and their inability to grow on bacteriological media. When these organisms were inoculated into the foot-pads of mice, small infiltrates containing acid-fast bacilli developed.

The present communication describes our continued experience with the experimental disease in mice. Microscopic lesions have been regularly produced when the nasal washings contained acid-fast bacilli, and the incubation period has depended upon the numbers of organisms inoculated. The maximum number of mycobacteria produced in the foot-pad was usually limited to about 10^6, and multiplication has occurred when the inoculum has contained an appropriately small number of bacilli. The oldest strain has been through four passages.

Materials and Methods

Most of the specimens were collected at the United States Public Health Service Hospital at Carville, Louisiana, but some were taken at the Central Luzon Sanitarium at Tala, near Manila. In brief, the nasal passages were washed with 500 ml. of balanced salt solution and the bacilli concentrated by centrifugation. Bovine plasma albumin was added to a concentration of 1 per cent and shipment made under wet ice refrigeration to the Montgomery laboratory, where decontamination was carried out by treatment with NaOH, and the bacilli were again spun down. This time they were resuspended to a measured volume (usually 2 ml.). Microdrops (2 μl.) were placed on a slide, and the number of bacilli estimated after the careful examination of a strip across the equator of the drop. Modifications since the previous description (1) are the dilution of the aliquot to be counted with an equal volume of formol-milk (2), and the covering of the drops with gelatine-phenol (2) before staining. It has been found important to minimize heating during fixation of the microdrops to the slide. Staining

with carbolfuchsin is currently done at room temperature for 20 minutes, since this gives brighter staining of the bacilli than does steaming for 5 minutes. The ratio of the area of the strip examined to the area of the microdrop is D/1.27 in which D is the diameter of the drop expressed in microscope fields. At least 2 drops were counted for each preparation. The lower limit of detectability is about 3×10^4 bacilli in the 2 ml., or 4.5×10^2 bacilli in the 0.03 ml. of inoculum. The figures given for low dosages are subject to considerable sampling error. Figures given for dosages less than this minimum were obtained by the "complete" method (1), that is, the entire drop was examined.

Inoculation of mice was done superficially in the right hind foot-pad with 0.03 ml. This amount balloons the skin and distributes the inoculum longitudinally. Usually 20 mice were inoculated with the same material. They were kept in a room at 70°F., and sacrifices were usually usually done at monthly intervals. The foot was removed, a slit cut in the dorsum to aid penetration of the fixative, and the foot fixed for at least 3 days in neutral formalin. Decalcification was carried out with frequent changes of 5 per cent formic acid in 70 per cent ethanol. About 20 days were required at room temperature, or about 7 days at 37°C. Decalcification at 45°C. is more rapid, but the nuclei no longer stain normally. Many of the decalcification procedures that are said to be rapid have given a poor result, presumably because the technical requirement is particularly high when the lesion to be examined is located peripherally under the skin, and the bone to be decalcified is in the center. Sections were cut 6 to 8 μ thick, hematoxylin, azure, and eosin stains were performed by the Harris procedure, and acid-fast stains by the Fite-Faraco technique.

Harvests.—The foot was washed in soap and water, rinsed, dried with sterile gauze, and the foot-pad carefully cut off with scalpel and forceps. The tissue was then minced well with sharp scissors, placed in the cup of a Mickle tissue disintegrator containing about 20 3 mm. glass beads, and 2 ml. balanced salt solution were added. One minute of vibration was used with an amplitude of 5 mm. Such treatment in the disintegrator had been found not to decrease the colony count of a suspension of *Mycobacterium tuberculosis*, and it did not affect the viability of the more fragile *Pasteurella tularensis* (3). Bovine albumin was then added to a final concentration of 0.1 per cent. The remaining clumps of tissue were removed by allowing the preparation to settle in a test tube for 2 minutes, then removing the supernate and making it up to volume. Sometimes this had to be repeated. The volumes were recorded at each operation and taken into account in calculation of the final figure, the number of acid-fast bacilli harvested per foot.

Passages were scheduled after examination of the sections revealed a "significant" lesion, defined as one occupying at least one-quarter of the microscope field with 12 × 60 magnification. After the mouse was sacrificed usually 2 to 3 months passed before the sections could be completed, examined, and the passage performed. Recently it has been possible to shorten this to about 1 month. To minimize the effects of variation among mice, 4 were sacrificed for harvest if possible and their foot-pads pooled and processed together.

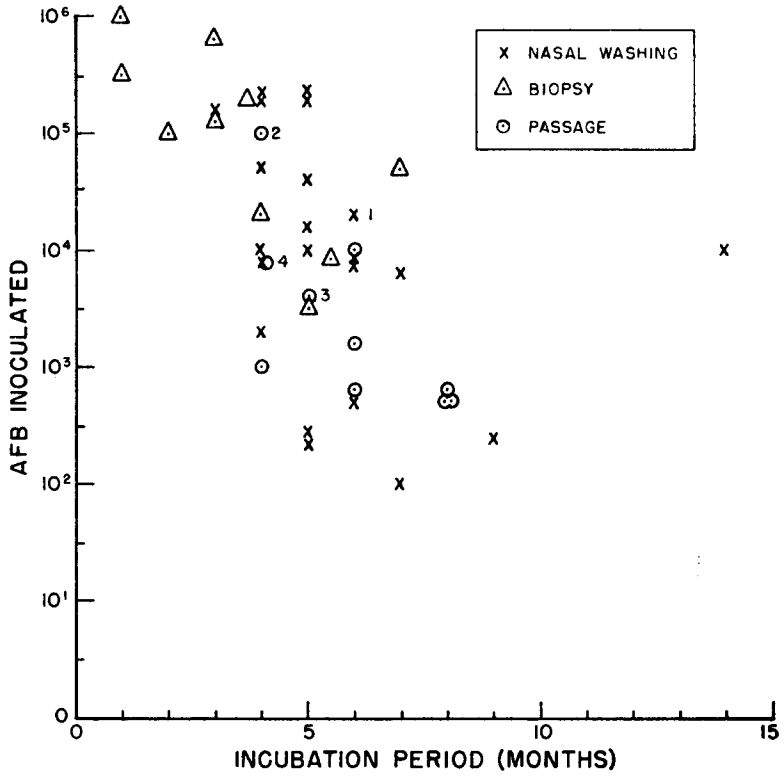
The biopsies were all collected at Carville, and without the addition of fluid they were shipped to the Montgomery laboratory where they were treated according to a procedure similar to that used for foot-pads. Usually about 0.1 gm. was processed.

Materials to be inoculated were kept in an ice bath. Infectious precautions need to be observed throughout and most of the operations, including the animal work, have been carried out in an infectious disease hood.

RESULTS

Microscopic Anatomy.—The lesion in the mouse foot was only irregularly observable with the naked eye, and its evolution has been followed by examination of sections.

The distribution of the inoculum could be seen in sections of some mice sacrificed the next day if large numbers (more than 10^5) of bacilli were present. Usually the bacilli were concentrated near the groups of vessels and nerves that overlie the muscle bundles, or they were spread out subcutaneously. Less frequently they could be found in the fascial separations in the muscle bundles, and in C57 mice they some-

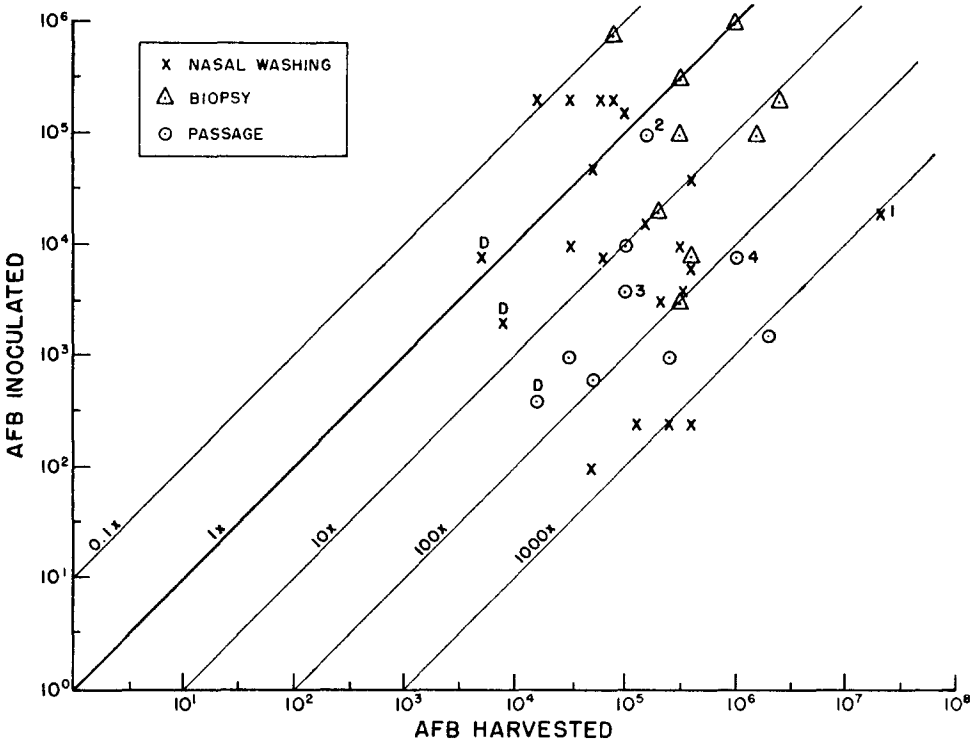


TEXT-FIG. 1. The relationship between the number of acid-fast bacilli (AFB) inoculated and the incubation period (time before development of a "significant" microscopic lesion).

times extended between the metatarsal bones to the dorsum. The lesions that developed later conformed to this distribution and showed little evidence of spread.

The time of appearance of the lesions depended upon the number of acid-fast bacilli in the inoculum (Text-fig. 1). Lesions developed regularly in 1 to 2 months with inocula of $10^{5.5}$ to $10^{6.0}$, and these were probably not associated with increase in the number of bacilli. With inocula of about 10^3 acid-fast bacilli neither infiltrates nor acid-fast bacilli were seen until about 6 months. Those seen first consisted at times of only a few cells containing acid-fast bacilli. Characteristically there was an increase in the size of the infiltrate and the number of bacilli in the following months until one or more 12 × 10 microscopic fields were occupied. Typical lesions are illustrated in

Fig. 1 to 4. There was no necrosis even in the largest lesions. Most of the cells in the infiltrate were large round cells, that is, macrophages and epithelioid cells, but lymphocytes were occasionally numerous. Polymorphonuclear leucocytes were rare. Although often near the lesions, the nerves were not observed to be involved. Most of the bacilli appeared to lie within the cells and large intracellular clumps were



TEXT-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 numbers of bacilli; those below, increases. The two marked *D* were associated with unfavorably delayed harvests. Those numbered *1* through *4* indicate the successive passages of the oldest strain, N2366.

not uncommon. The bacilli were frequently solidly and brightly stained in the early lesions and in some of the older lesions, too. The older lesions more frequently contained bright, beaded organisms, and occasionally pale staining bacilli were seen in the oldest lesions. Over-all there was an orderly progression to be observed in the sections taken from month to month, but there was considerable variation both in the extent of the infiltrate and number of acid-fast bacilli.

The evolution of lesions following inoculation of passage material was not different from that produced by primary inoculation of nasal washings or of

biopsy material when the dosage of acid-fast bacilli in the inoculum was taken into account.

Relationship between Number of Acid-Fast Bacilli in the Inoculum and Incubation Period.—This is shown in Text-fig. 1 for an incubation period defined as the time until the development of a "significant" lesion. With one exception the plotted points fall in a well defined area. It is of particular interest that the passage material still falls in this area, since this indicates that the bacilli in passage are still *M. leprae.* The tendency for the biopsy values to group in the upper part of the figure, and the passage values in the lower part is a reflection of the greater yield of bacilli from the human than from the mouse tissues, and also of our former practice of avoiding dilution of the inoculum.

Relationship between the Number of Acid-Fast Bacilli in the Inoculum and the

TABLE I

Consistency with which "takes" are produced according to source of inoculum

Source of inoculum	No. of Acid-fast bacilli inoculated	No of "takes"
		No. of materials inoculated
Nasal washings	$<4.5 \times 10^2$	0/16
Nasal washings	9×10^1 to 2.2×10^5	22/22
Biopsy	9×10^1 to 9.4×10^5	12/16
Passage material	4.5×10^2 to 1.1×10^5	10/11

Number Harvested at Time of Passage.—This essential correlation is shown in Text-fig. 2. Points falling above the heavy line marked *1X* represent decrease in numbers of organisms; those below, an increase. The distribution of points indicates that the number of acid-fast bacilli produced in the foot-pad is usually limited to about $10^{4.5}$ to $10^{6.0}$. All of the data, except those obtained very recently, have been plotted in this figure, and the three unfavorable points marked *D* are associated with delayed harvest; that is, the harvest was carried out 4 to 8 months after the first observed "significant" lesion had developed. These 3 occasions excepted, harvests have regularly given increases in numbers of bacilli of 50- to 1,000-fold when inocula contained $10^{2.0}$ to $10^{3.5}$ acid-fast bacilli. At present, materials are diluted if possible to contain about 10^3 organisms in the 0.03 ml. before inoculation.

The passage materials have not behaved with significant differences from the original clinical material.

Consistency with Which "Takes" were Produced According to Source of Inoculum.—This is shown in Table I. A "take" is defined as the production of a "significant" lesion. Such lesions have resulted in each of 22 instances where the nasal washing was observed to contain acid-fast bacilli. They have not

been seen in 16 instances where the nasal washings were not found to contain acid-fast bacilli; these have been followed for 10 to 18 months. The inoculation of acid-fast bacilli from biopsy material has produced lesions in 12 of 16 groups of mice. The results of 11 passages are complete. All originated from nasal washings, and results of passage of biopsy material are not yet available. Passage was successful 10 times. The reason for the single failure is not known, and although sections were available from only 5 mice, they were sacrificed at an opportune time; *viz.*, 6 to 8 months.

Results with Passages.—Nine of the 10 successful passages are plotted in Text-fig. 2. Strain N2366 has successfully completed 4 passages with a total increase in numbers of bacilli of 3.8×10^6-fold (numbered *1*, *2*, *3*, and *4*, in Text-fig. 2). Strain N2387 has completed 3 passages with a total increase of 4.1×10^4-fold. The other 5 strains have completed only 2 passages with total increases in numbers of acid-fast bacilli of 9- to 2300-fold. The amount of increase in all passages has been determined principally by the relationship shown in Text-fig. 2, and fold-increases have been greater when the inoculum was small.

Rate of Multiplication.—The 4th passage of N2366 provided data favorable for the estimation of the rate of multiplication. The inoculum of 7.6×10^3 bacilli and the harvest of 9.6×10^5 at 7 months, corresponds to a generation time of 0.86 months. Other recent data from mice inoculated with small numbers of organisms, also obtained under optimal conditions for counting and in prompt harvests, gave generation times of 0.93, 0.88, and 0.85 months associated with harvests of 1.0×10^6, 1.4×10^6, and 1.1×10^6, respectively. Of these 3, 1 was the 3rd passage of N2387, and 2 were primary biopsy inoculations.

Ability of the Bacilli to Withstand Shipment.—From the time of collection of the nasal washings and biopsies at Carville until the mice were inoculated in Montgomery, about 24 hours usually elapsed. The Philippine specimens, however, were delayed and about 100 hours had passed before inoculation. The shipments were made in vacuum bottles which were reiced twice en route, and they were received well iced at their destination. Of the 8 Philippine specimens, 6 contained acid-fast bacilli and the disease that followed their injection did not differ from that caused by the Carville specimens. The position of the 6 positive specimens in Text-figs. 1 and 2 was not unusual. Thus there was no evidence that the bacilli deteriorated en route. The length of time elapsed would allow shipment under usual conditions from any part of the world near air routes, if reicing could be arranged along the way.

Other Types of Inoculation.—C57 black mice were inoculated in parallel with the CFW mice in an extensive comparison. Not much difference was observed in microscopic sections or in acid-fast bacilli harvested. Since the C57 strain was extensively infected with *Salmonella* and it offered no advantage, its use was discontinued. Both CFW and C57 mice were also inoculated intratestic-

ularly in another extensive comparison. Infiltrates containing acid-fast bacilli resulted, but they did not progress to the extent of the foot-pad lesions. Although with some inocula the number of bacilli harvested from the testicles was as high or somewhat higher than that from the foot, it was very much lower with other inocula.

Syrian golden hamsters have also been inoculated in the foot-pad and testicle in another large comparative study. They have been followed for periods up to a year, and this was longer than that needed to see that mice inoculated in parallel with the same material responded typically. The foot-pad lesions were less extensive than those produced in mice, and no lesions have been observed in testicles. Harvests of hamster testicles have not revealed increase in number of bacilli. In one experiment, positive nasal washings from the same three patients were inoculated into the foot-pads of mice and into the testicles of hamsters. At 5 months, harvests revealed increases in numbers of bacilli in the mouse foot-pads, as usual, but there were decreases in the hamster testicles. Passages to mouse foot-pads of these harvests resulted in the characteristic picture in the case of passage of mouse foot-pads, but no disease resulted from passage of the hamster testicle material. Chinese hamsters and Mongolian gerbilles have also been inoculated in the foot-pad and although microscopic granulomas were produced they were not as extensive as those in the mice, nor were there so many acid-fast bacilli present.

Inoculation of Bacteriologic Media and Tissue Cultures.—Most of the materials inoculated into mouse foot-pads and harvested from them have been inoculated onto bacteriological media. For the last year this has included Loewenstein-Jensen medium, 25 per cent blood agar, 7H9 agar with oleic acid–albumin supplement, and the same as broth. Only non-acid-fast organisms have been grown, and these were the expected varieties of surface contaminants. Most of the same materials have also been inoculated into cultures of HeLa cells (4). Coverslips fixed at intervals up to 2 months have not revealed evidence of mycobacterial growth, although the organisms could be visualized in the cells for much of the period.

DISCUSSION

The isolation of an infectious agent from disease does not, of course, establish that the agent causes the disease, and it falls to the investigator to clarify the relationship. In general, the evidence for the etiologic position of the agent depends upon its properties. In the present case inability to cultivate the organism has prevented recourse to "Koch's postulates." Neither has it been possible to resort to serologic methods after the manner used to explore the role of viruses and rickettsiae in infectious disease.

The evidence that indicates that the acid-fast bacterium here described is the organism that causes human leprosy is as follows: (*a*) "Takes" in mice were

observed in most instances following the injection of acid-fast bacilli from patients with leprosy. Some bacilli from biopsies failed to produce the experimental disease, but it is possible that these bacilli were not viable (see below). (*b*) "Takes" were not observed following the injection of nasal washings not known to contain leprosy bacilli. The negative nasal washings were obtained chiefly from patients with treated lepromatous leprosy, and from tuberculoid and border line leprosy, described in (1). (*c*) Quantitation of the acid-fast bacilli showed a regular relationship between the number of bacilli inoculated and the experimental disease in mice. This was manifested by the dose-incubation period relationship (Text-fig. 1) commonly seen in experimental infections, and by the inoculum-harvest relationship (Text-fig. 2). These quantitative results are compatible with the notion that the acid-fast bacilli multiply with a generation time in the neighborhood of 0.9 months, until they reach a total of about $10^{4.5}$ to $10^{6.0}$ bacilli. Disease is then detectable histologically and futher bacillary increase does not seem to occur. (*d*) Continued passage through mice has not altered these quantitative relationships. (*e*) In the preceding paper (1) a relationship was described between the clinical manifestations of leprosy and the number of acid-fast bacilli, specifically, the number of acid-fast bacilli recovered in the nasal washings was correlated with the clinical severity of the lepromatous disease. The quantitative aspects of the two papers then, serve to correlate the clinical manifestations of the natural disease to the experimental disease in mice. (*f*) The microscopic anatomy of the experimental disease seems compatible with human leprosy. A granuloma consisting chiefly of large round cells is observed in mice, and there is no necrosis. The organisms appear to be chiefly intracellular. (*g*) Materials producing "takes" in mice have given negative results on bacteriological media. Although this in negative evidence, it does tend to rule out a very large number of known mycobacterial species that are easily grown on the media used. Similar considerations apply to the tissue culture results, which have been obtained with methods suitable for the cultivation of many mycobacterial species (4). (*h*) It seems possible to rule out the murine "leprosy" of Stefansky on the basis that the experimental disease here seen in mice is more limited and more slowly progressive.

It had been proposed that *M. leprae* in the nasal washings might be a more reliable source of viable organisms since they were being continually excreted (1). In comparison, *M. leprae* in skin, because they typically lie in closed lesions, might represent a storage of non-viable structures. Positive results with skin organisms, however, have been obtained in 12 of 16 instances. Only 1 of the negative instances occurred with skin bacilli from a patient whose nasal washings contained acid-fast bacilli. The other failures with leprosy bacilli from skin were seen with patients with negative nasal washings, and who might, therefore, have received sulfone treatment (1). The results obtained to date do not allow an estimate of the relative numbers of viable bacilli in skin *versus* nasal washings.

The experimental mouse disease reported here does not resemble closely that reported by Chatterjee (5), which was a massive generalized infection. The hybrid strain of mice used by Chatterjee has recently become available to us, and it is being compared to the CFW strain. The foot-pad lesions, which are now appearing, are not much different from those in the CFW mice inoculated in parallel, and the first harvests show no important difference in numbers of acid-fast bacilli. It has not been possible to obtain mouse passage material from the Indian laboratory for comparison. The mycobacteria of testicular granulomata in hamsters reported by Binford (6) differ in several aspects, notably their cultivability. Evidence for difference was also obtained the the experiment described above, where material that could be passed from mouse foot-pads could not be passed from hamster testicles.

The experimental disease in mice has proved reasonably consistent to date. It appears capable of detecting small numbers of bacilli, just how few is not yet known. It would seem likely that it could serve for many purposes, for example, for chemotherapeutic tests and for studies of immunity and vaccination, and efforts are being made in these directions.

SUMMARY

When leprosy bacilli from human patients are inoculated into the foot-pads of CFW mice, a microscopic granuloma containing acid-fast bacilli develops in a characteristic manner. This has been seen in 22 of 22 instances with leprosy bacilli from nasal washings, in 12 of 16 instances with leprosy bacilli from skin biopsies, and in none of 16 cases where the nasal washings were not observed to contain leprosy bacilli.

Quantitative studies revealed a relationship between the number of bacilli inoculated and the time required for the appearance of the lesions. The incubation period was usually 1 to 2 months when the dose was $10^{5.5}$ to $10^{6.0}$ bacilli and about 6 months when the dose was about 10^3 organisms.

After the development of the lesion, the number of bacilli harvested was usually in the range $10^{4.5}$ to $10^{6.0}$, regardless of the number inoculated. When the inoculum has contained $10^{2.0}$ to $10^{3.5}$ acid-fast bacilli, and harvests were reasonably prompt, there were regular increases of 50- to 1000-fold.

Passage to new groups of mice has been successful 11 of 12 times. Most of these were second passages. One strain has been maintained in 3 passages with a total increase in acid-fast bacilli of 4×10^4-fold. Another strain has been through 4 passages with a total increase of about 4×10^6-fold.

Cultures on bacteriological media favorable for the growth of most known mycobacterial species have not shown growth of mycobacteria.

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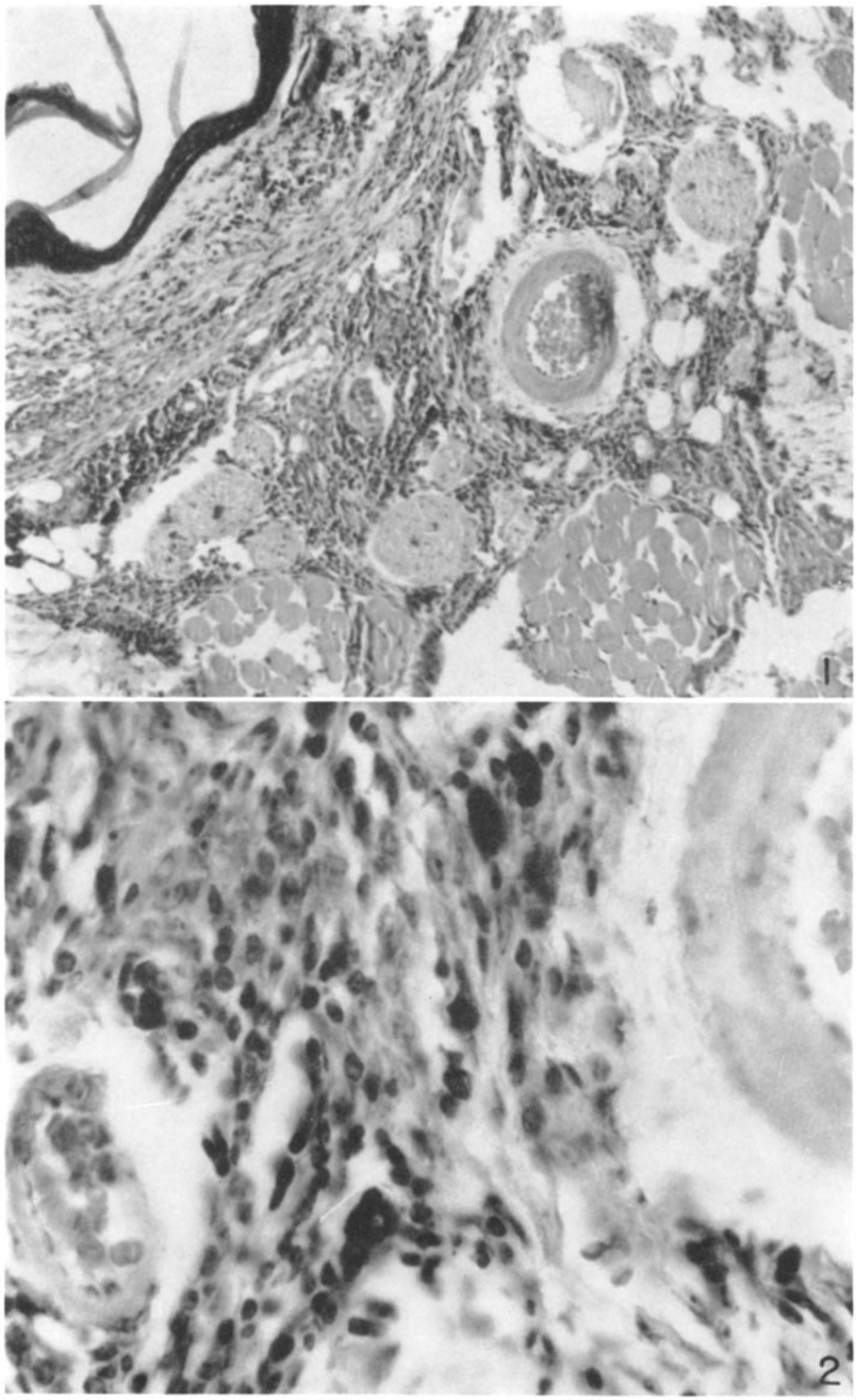
EXPLANATION OF PLATES

PLATE 38

FIG. 1. A well developed granuloma in mouse foot-pad. The infiltrate centers around the vessels and nerves, which lie in a groove on the muscle bundles, and it extends into the subcutaneous tissues and between the muscle bundles. It also reached across several contiguous microscopic fields. (Hematoxylin, azure, and eosin stain, × 190.)

FIG. 2. A high power view of the center of the same section. Just above the center are some large mononuclear cells with generous amounts of (pink) stained cytoplasm. These cells typically contain acid-fast bacilli. (Hematoxylin, azure, and eosin stain, × 840.)

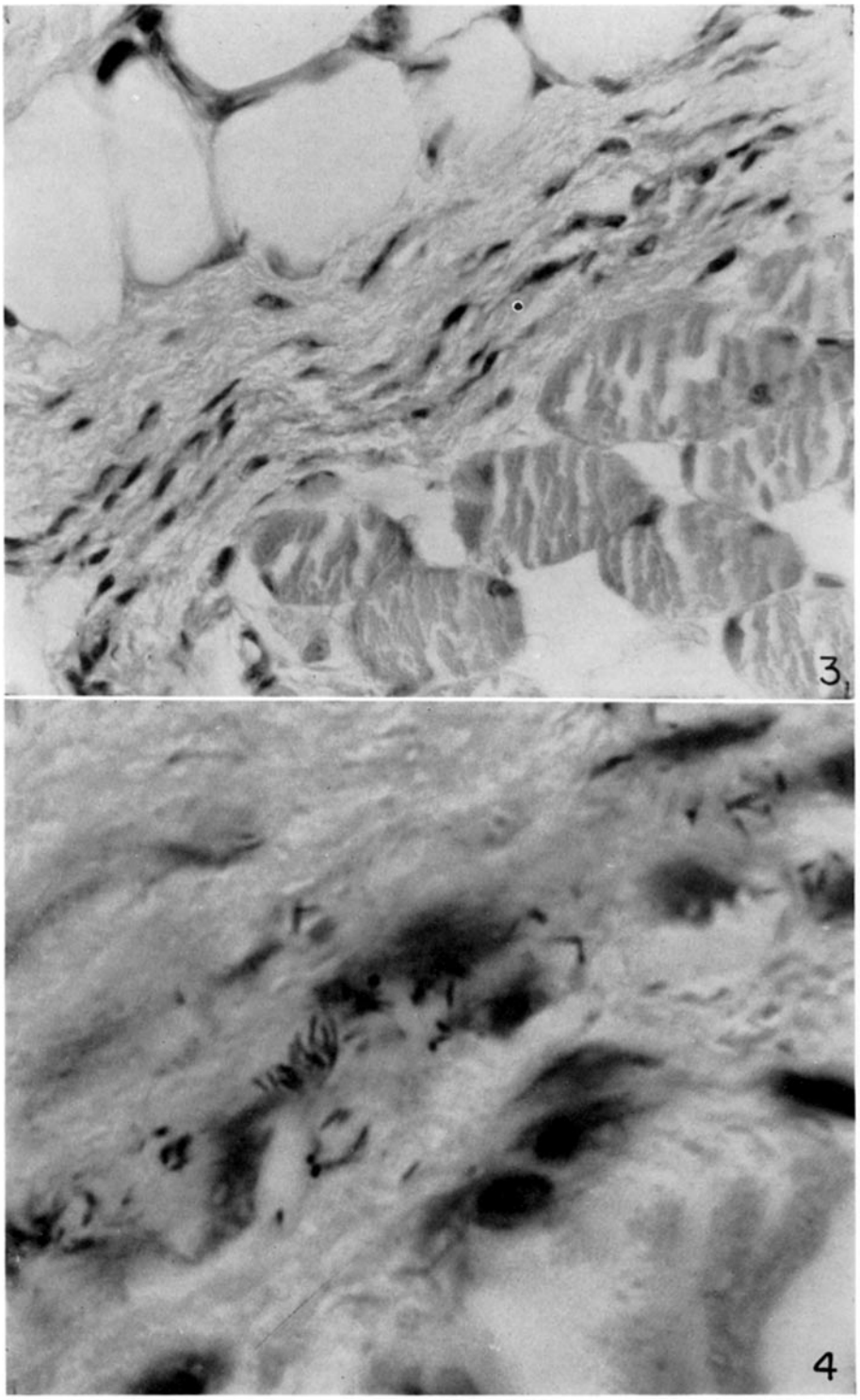


(Shepard: Human leprosy bacilli injected into mice)

PLATE 39

Fig. 3. A less extensive and diffuse infiltrate, which is largely subcutaneous. Such disease is sometimes difficult to detect except in acid-fast-stained sections. (Hematoxylin, azure, and eosin stain, × 750.)

FIG. 4. Acid-fast stain of another section of the same lesion. Many acid-fast bacilli are centered about the nuclei. (Fite-Faraco stain, × 2600.)



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