

# EXPERIMENTAL INOCULATION OF HUMAN LEPROSY IN LABORATORY ANIMALS<sup>1, 2</sup>

## III

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The authors have conducted experiments for the last 5 years with the transmission of leprosy from human lesions to small laboratory animals, such as hamsters, rats, and mice. In two previous papers of this series (<sup>1, 2</sup>) we have reported on our work up to the end of 1961. By way of introduction to this third paper, we shall refer briefly to the fundamental ideas of our experiments, although they were set forth fully in the first article.

Our working hypothesis differed from that of other investigators in that we considered the nature of the inoculum to be as important as, or even more important than, the condition or preparation of the animals, to which previous workers, notably Adler, Binford, Chatterjee, and Bergel, paid special attention. These investigators considered it essential at the same time to use inocula with the greatest number possible of bacilli. This concept naturally eliminated all but lepromatous sources. Of the four workers named, Adler conditioned his animals by splenectomy, Chatterjee took advantage of the special susceptibility of a strain of black mice produced by cross-breeding the gray and white varieties, and Bergel used a pro-oxidant diet to induce changes in the metabolism that would lower their resistance. Binford selected the parts of the body with lowest temperature for his inoculations. On that principle Shepard produced lesions in the footpads of mice. It was the principle to which we also adhered in our experiments.

While we used the hamster preferentially in the 2,502 animal inoculations that we have made to date, because it is Mitsuda-negative, 40 per cent of the animals used were of other species, such as rats, mice, guinea-pigs, rabbits, swine, and even a few fishes.

We did not resort to the use of cortisone, nor of x-rays, as a supposed means of lowering the resistance of our animals. Except for ordinary precautionary sterilization, no method of treatment or preparation was applied to the site of inoculation. While most of the animals were inoculated in the ears, we used also the subcutaneous and intraperitoneal routes, and some of the hamsters were injected in their cheek pouches.

Our basic hypothesis as regards the inoculum may be stated as follows: The form of *M. leprae* in lepromatous lesions is evidently a

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polar type that has evolved from an unstable precursor to form a stable strain so well adapted to the intracellular medium of the host cells that it multiplies unhindered and dies of the fatty degeneration of old age. As a matter of fact, electron microscopic studies have shown that the vast majority of the bacilli in lepromatous lesions are degenerated, in the course of disintegration, and probably dead. They have seemed to be the form least likely to live and multiply in the cutaneous cells of another species. On the other hand, the bacilli present in borderline lesions, though few, could be considered to represent a genetically unstable strain capable of evolving either into the stable, prolific polar strain of lepromatous leprosy or into the short-lived polar strain of the tuberculoid form. It seemed reasonable to suppose that such a transitional type would be the one most likely to evolve into a strain adapted to the intracellular environment of a new host. This concept was strengthened by electron microscopic study, which showed that the bacilli of borderline origin were intact and seemingly healthy, in sharp contrast with the degenerate aspect of those from lepromatous lesions.

From 1959 to the middle of 1962 no lesions were produced in any of our animals inoculated with bacilli of lepromatous origin. However, in the hamsters inoculated with bacilli from borderline lesions results were obtained after an incubation period of 8 months. As passages were made in successive groups of hamsters, it was observed that the period of incubation was shortened, with each successive passage, until it became less than four months in the eighth passage. The shortest incubation period observed was in the fifth passage, where lesions were observed 55 days after inoculation.

Repeated attempts to cultivate the strain of borderline origin developed in the hamster, gave negative results in the Löwenstein-Jensen medium.

In the latter half of 1962 we obtained, for the first time, lesions in a group of hamsters inoculated with bacilli from a lepromatous source. From these lesions a culture was obtained of an acid-fast mycobacterium in the Löwenstein-Jensen medium, in contrast with the complete failure to obtain a culture from the hamster lesions of borderline origin. The strain was classified as a nonphotochromogenic mycobacterium. It appeared on the surface of the medium three weeks after the medium was inoculated, in the form of small, slowly growing, grayish colonies measuring 3-10 mm. in diameter and numbering 10-12 in each tube. They became yellow with age, regardless of whether or not they were exposed to light. They showed the following characteristics by biochemical tests:

- Homogenization test: positive
- Test with Congo red: negative
- Cord formation: negative
- Catalase content: strongly positive
- Konno niacin test: negative

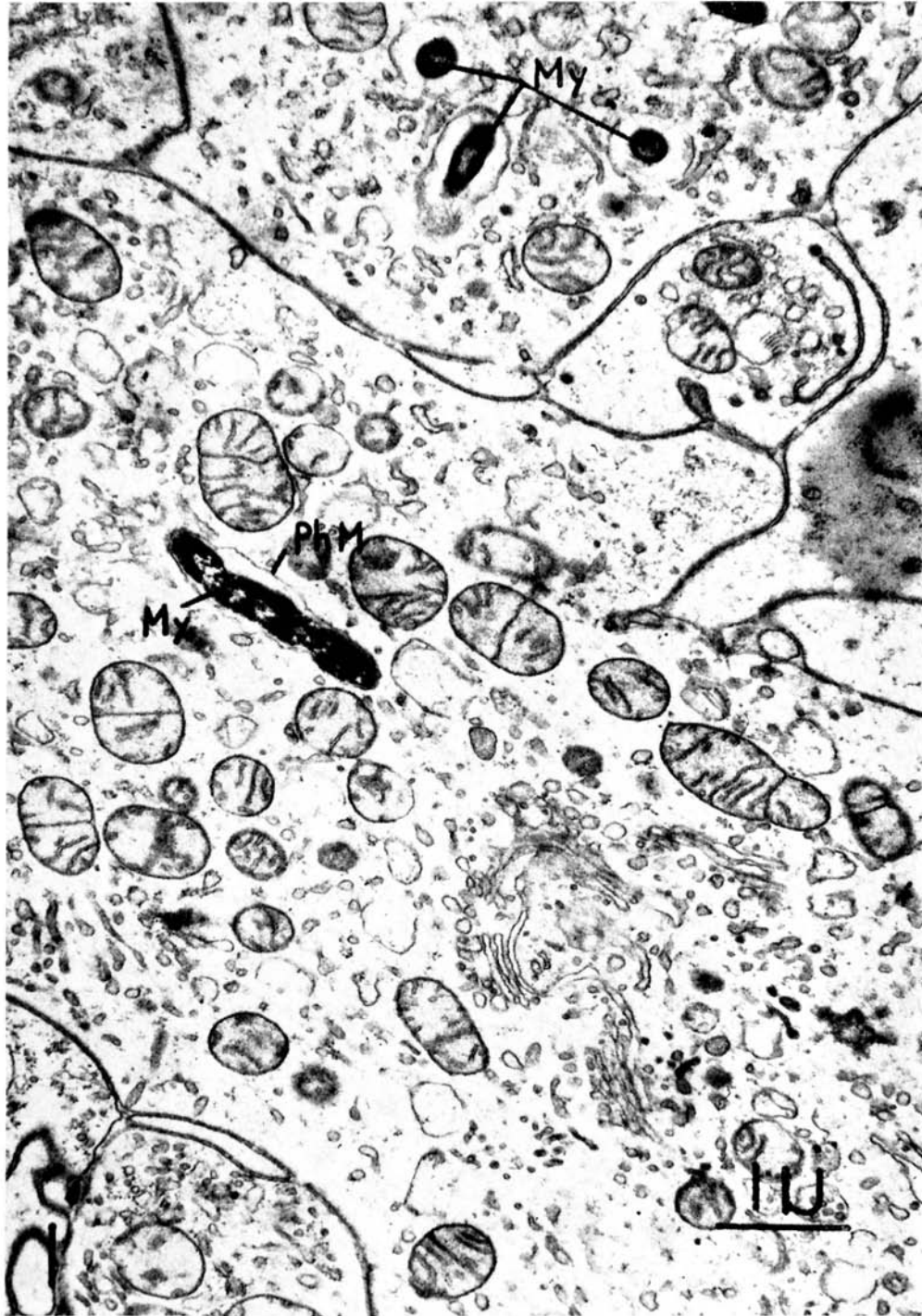


FIG. 1. In host cells bacilli (**My**) are enclosed by a membrane (**PhM**), presumably representing phagocytic membrane. No remarkable changes occur in the host cell cytoplasm.

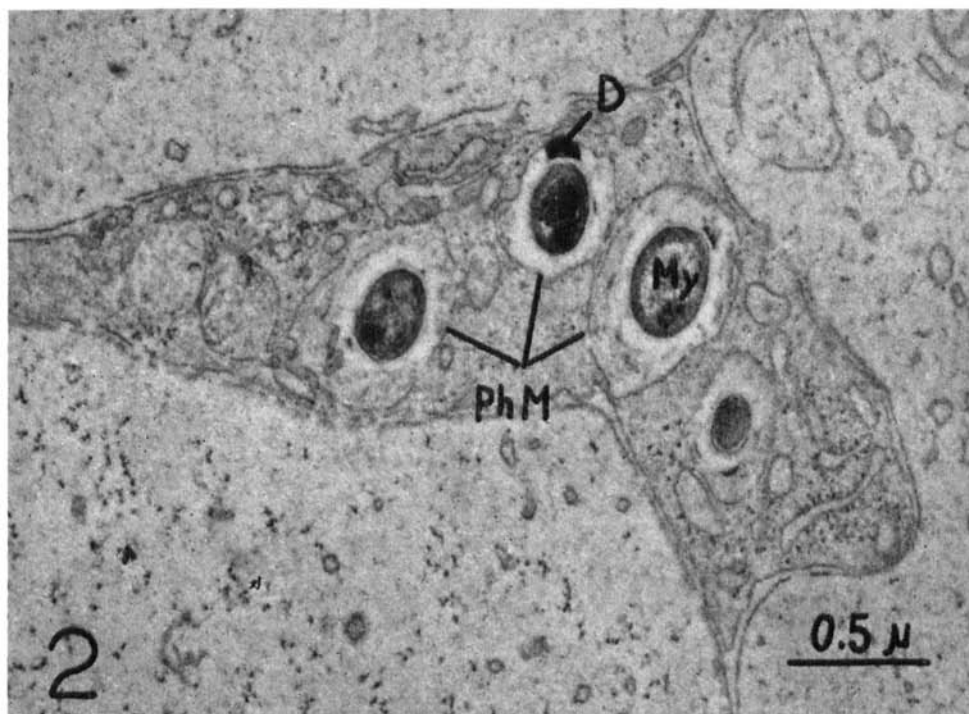


FIG. 2. Bacilli in an immature reticular cell in a lesion. The amorphous or fibrous substance surrounds the bacilli (**My**) within the phagocytic membranes (**PhM**). Note a dense droplet (**D**) attached to the bacillus.

The strains of *M. leprae* developed in the hamsters from borderline and lepromatous human strains and the cultivable form of lepromatous origin differ also in their cytoplasmic structures and in their immunologic properties.

The three groups involved have been studied by the electron microscope. Micrographs grouped as follows show the bacilli and intracellular aspects of their host cells.

*Group I.*—This comprises the noncultivable strain developed in the hamster from borderline lesions poor in bacilli. In contrast with the paucity of the numbers of its precursors in human lesions, it is highly prolific in the tissue of the hamster (Figs. 1 and 2).

*Group II.*—The micrographs of this group represent lesions produced in the hamster with bacilli from lepromatous human lesions as precursors (Fig 3).

*Group III.*—We have here the cultivated but still pathogenic form of the bacillus of lepromatous origin developed in the hamster. The macroscopic aspect of its colonies on the Löwenstein-Jensen medium, and its characteristics as revealed by biochemical tests, were dealt with elsewhere in this report (Fig. 4).

The macroaspect of the viscera of hamsters infected with the strain of borderline origin is shown in Figures 5 to 7 inclusive, and a smear from a splenic lesion is shown in Figure 8.

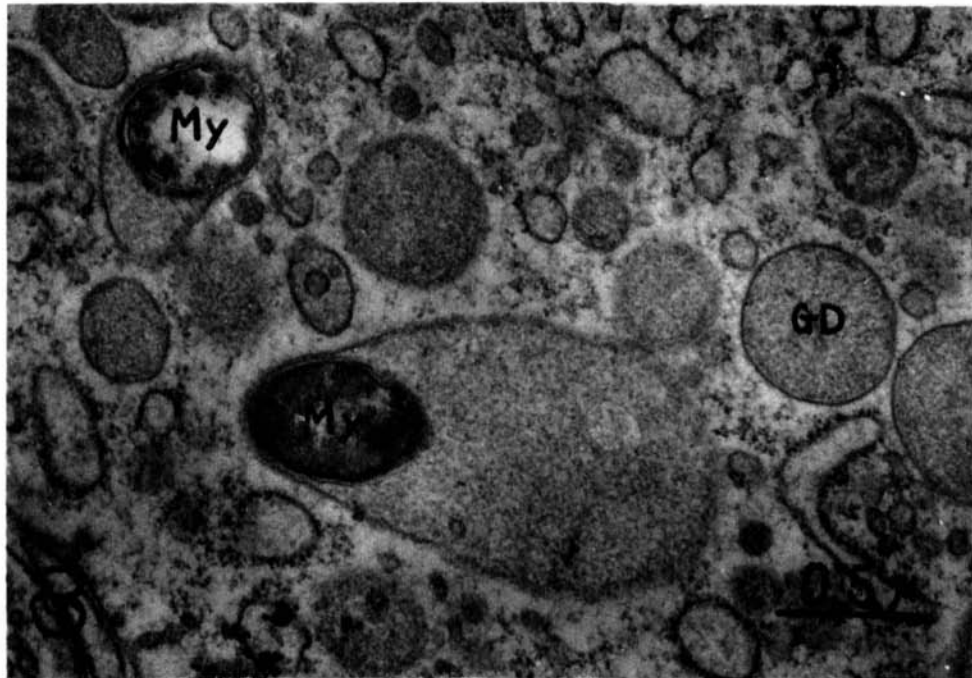


FIG. 3. Moderately dense droplets (GD) consisting of fine granular matrix are distributed in the host cell cytoplasm. Sometimes the bacilli (My) are embedded in these droplets.

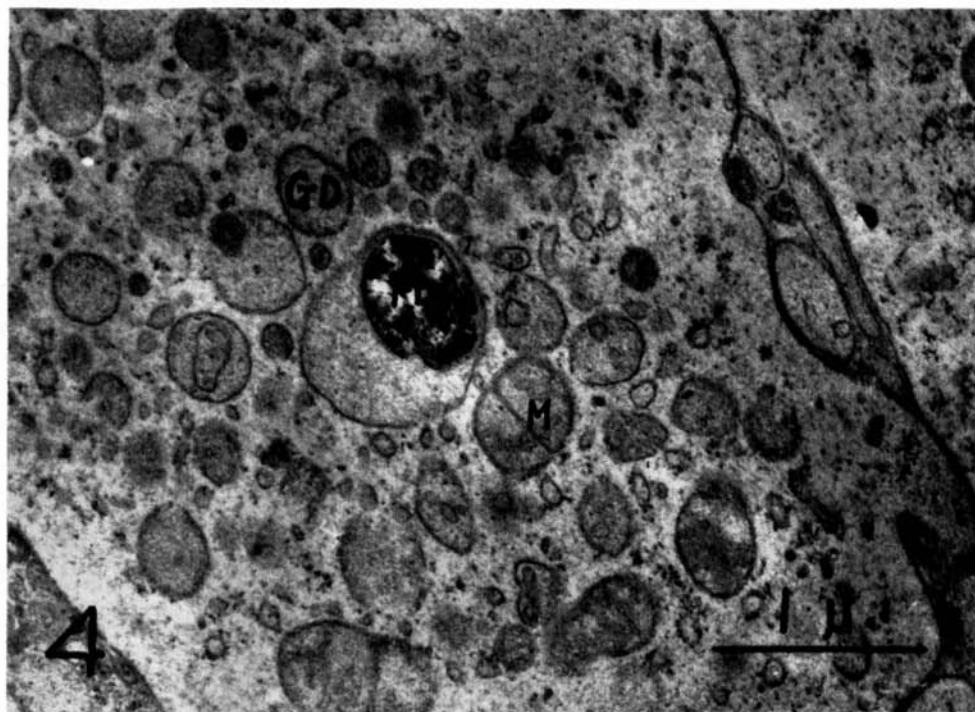


FIG. 4. Moderately dense droplets (GD), similar to those in Figure 3, are seen enclosing the bacillus (My). These droplets are distinguishable from mitochondria (M) because of the lack of a typical double membrane.



## IMMUNOLOGIC ASPECTS

The lesions produced in the hamster by the strain of borderline origin were used for the preparation of an antigen for intradermic immunologic tests. These tests were carried out only with lepromatous patients hospitalized in the Cabo Blanco Antileprosy Sanatorium and under constant careful observation and control. Forty-two patients took part in the experiment. A larger group could not be obtained under present conditions in the Sanatorium.

For the purpose of comparison the bacillary antigen from the lesions in the hamster was administered jointly with lepromin from human lepromatous lesions. Each antigen was prepared by the trypsinization technic in a manner similar to the preparation of bacilli for inoculation. As a preservative we used only 0.2 ml. of phenol. In each antigen the concentration was 45 million bacilli per dose.

In one group of patients intradermic injections of 0.1 ml. of each antigen were given simultaneously, the preparation from hamster lesions on the left and the human lepromin on the right forearm. In another group the human lepromin was administered one month after the antigen from hamster lesions.

The 42 patients varied greatly, clinically and bacteriologically, ranging from patients without apparent lesions, but bacteriologically positive, to patients with lesions advanced to the stage LL2. The same phenomena were observed in each group.

In tests with the hamster antigen on the left forearm the majority of patients showed an erythematous infiltration at the site of the injection 48—72 hours after. In a few others this early reaction was not observed. After one week, however, all the patients began to develop erythematous nodules that grew slowly in size until in some cases they reached a maximum diameter of 20 mm. with a necrotic center.

In the test with human lepromin on the opposite forearm no reaction was observed during the first week, but shortly thereafter typical nodules began to appear in all the patients at the site of the inoculation. These nodules grew slowly until the end of the fourth week, when the following diameters were measured:

In 3 cases	2 mm.
In 6 cases	3 mm.
In 6 cases	4 mm.
In 10 cases	5 mm.
In 12 cases	7 mm.

Seven out of the 12 cases with nodules 7 mm. in diameter showed a necrotic center.

As we said before, this positivization of the Mitsuda reaction was the same whether the antigens were administered simultaneously or after an interval of one month in the case of the human lepromin.

In 11 of the patients the nodules resulting from the reaction to



FIG. 5. Cross-section of a highly bacillated and much enlarged spleen from a hamster 20 months after inoculation. A spleen from a supposedly healthy adult hamster is shown at the right for comparison.

the human lepromin were extirpated for biopsy. We can say briefly that the nature of these nodules was revealed in the histopathologic study as epithelioid and tuberculoid, with few acid-fast bacilli.

The most interesting observation was that the lepromatous patients, who had reacted positively to human lepromin after receiving the hamster antigen, were still positive reactors one year later, when they were given the human lepromin alone.

Figure 9 illustrates the reactions in our lepromatous patient I. M. M. The upper large nodule with central ulceration on the left forearm, which measured 45 x 17 mm., represents the reaction to 0.1 ml. of our hamster antigen of borderline origin (A.M.), with a concentration of 45 million bacilli in that dose. The nodule below it represents the reaction to our hamster antigen (T.R.), which is also of borderline origin and contains about the same number of bacilli per dose of 0.1 ml. This nodule measured 15 mm. in diameter and had a 10 mm. necrotic center. The letters A.M. and T.R., used to designate the antigens, are the initials of the borderline patients from whom the original material was obtained for inoculation into the initial groups of hamsters of the two series of passages made this far.

The erythematous nodule on the right forearm is the reaction to the human lepromin representing sensitivity evoked by the hamster antigens used on the left. It is a typical positive Mitsuda reaction and measures 7 mm. or perhaps slightly more in diameter.

The positivization of the Mitsuda test after administration of the



FIG. 6. Cross-sections of 4 spleens, of which No. 1 is highly bacillated. It was removed from a hamster that died 20 months after being inoculated in one of the testicles. No. 2 was removed from a hamster of the 7th passage that died 11 months after being inoculated in a testicle. Numbers 3 and 4 show spleens from supposedly healthy adult hamsters.

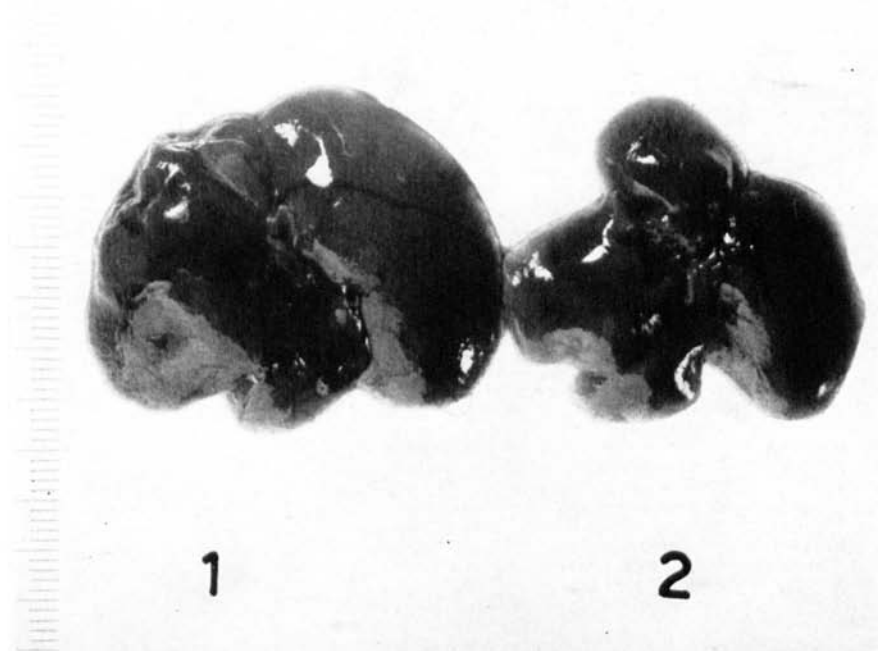


FIG. 7. Cross sections of 2 livers. The one at the left is from a hamster of the 7th passage that died 11 months after inoculation. It is somewhat enlarged and slightly bacillated. The liver illustrated at the right is from a supposedly healthy adult hamster.



hamster antigen has been observed only with antigens prepared by the trypsinization method. Antigens prepared by the Dharmendra method, in which the bacilli are floated on chloroform, were ineffective.

No antigen was prepared from the strain of the bacillus of lepromatous origin developed in the hamster, as only 3 passages had been made at the time and the material available was insufficient for tests in a new group of patients.

An additional immunologic test was made with an antigen from the lesions of rats infected with the Stefansky bacillus, *M. lepraemurium*. This antigen was prepared by trypsinization to remove tissue particles, as in the preparation of human lepromin, and the concentration was adjusted to our standard of 45 million per injection of 0.1 ml. Here again we had a difference in the immunologic properties. In the nine patients that took part in this experiment the antigen failed to evoke a positive reaction to the human antigen on the right forearm, although it did produce sizeable nodules, some necrotizing, where it was injected on the left arm.

Subsequently to these experiments we received from Dr. C. H. Binford, research pathologist of the Leonard Wood Memorial, two antigen preparations, one of which was from testicular lesions in hamsters and the other from a culture of a strain of *M. leprae* made by the Shepard and Kirsh technic in a tween-albumin medium. The orig-

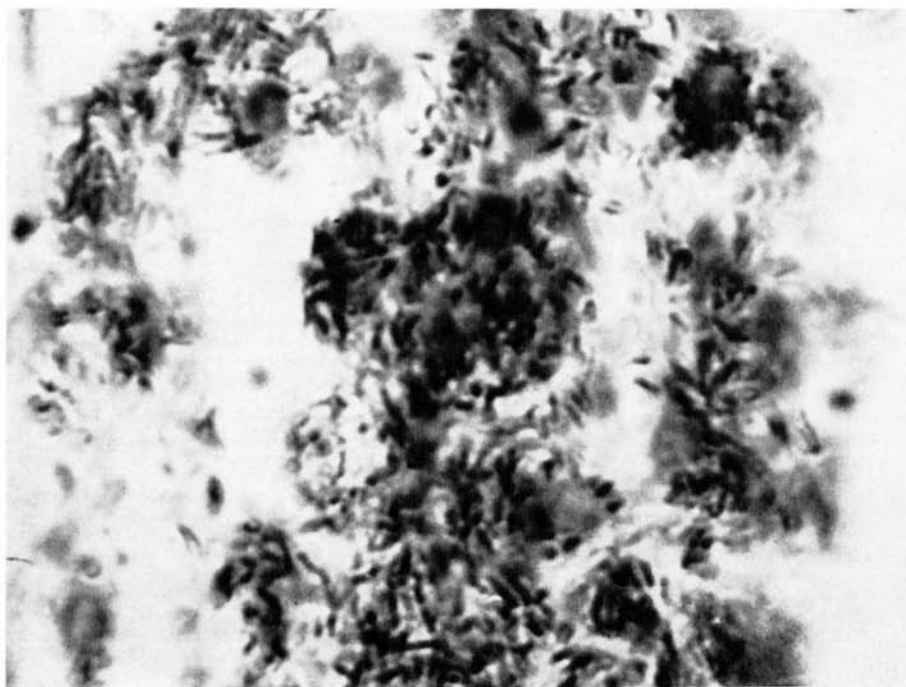


FIG. 8. Smear from a splenic lesion in a hamster of the 7th passage (Ziehl-Neelsen stain).

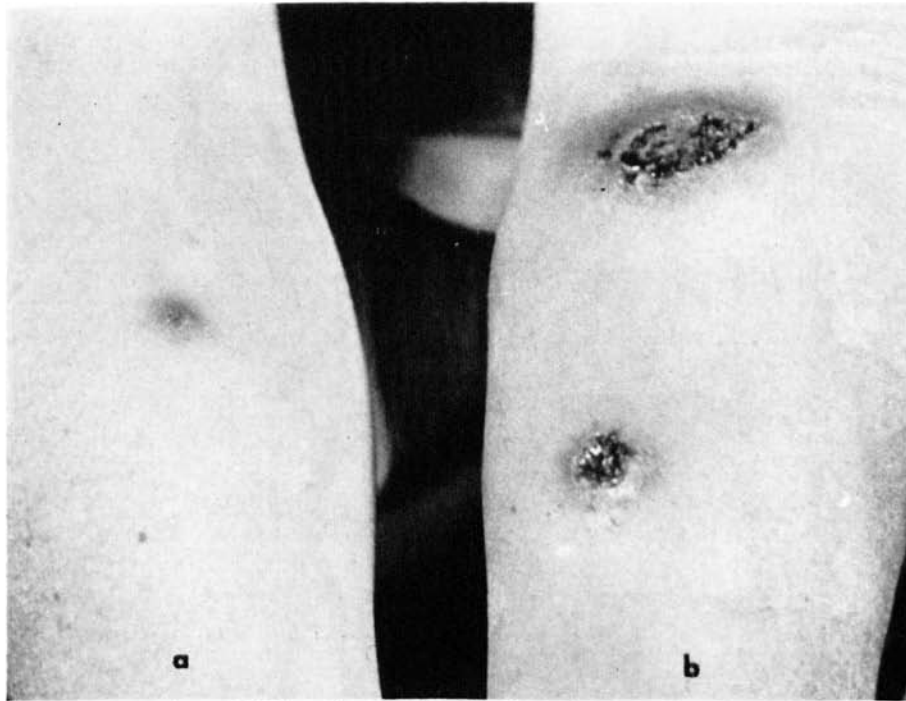


FIG. 9. Reactions of LL patient I. M. M. Left forearm (b) shows response to two hamster antigens, AM and TR, which evoked the positive reaction to human lepromin shown on the right arm (a).

inal mycobacteria in the case of each antigen were derived from lepromatous human lesions.

These antigens, which we have called the Binford antigens, in contradistinction to our own, were injected in two groups of lepromatous patients of five each. One group received the antigen from hamster testes, which Dr. Binford informed us had been prepared by the Wade technic for human lepromin, and the other received the preparation from the cultivated strain.

In both groups the reactions to the nonhuman antigens were similar to the reactions to our own nonhuman antigens, although in some of the patients they were somewhat less intense. Only one patient in the ten failed to produce a late reaction. The nodules of the other nine varied in diameter from 4 to 22 mm., and several had a necrotic center.

Our curiosity was aroused as to whether or not the Binford antigens, like one of ours, would evoke a positive sensitization to human lepromin. These antigens were accordingly given on the right forearm of all the patients. In nine of them a typical positive Mitsuda reaction was evoked by the third week. The diameters of the nodules, as observed at the end of the fourth week, were from 4 to 15 mm. in the group that received the antigen from testicular lesions, while in those evoked in the group that had received the antigen from the cultivated strain they measured from 4 to 7 mm.

## COMMENTS

A number of salient facts in these experiments are at present the objects of speculation as to their intrinsic nature, but they should be the objects of thorough investigation in the same respect. They may be summarized as follows:

1. The readiness with which we obtained lesions in the hamster when the animal was inoculated with material poor in bacilli from human borderline lesions.

2. Our continuous failure during nearly four years to produce lesions in the hamster with lepromatous human material rich in bacilli before we finally did get a positive result.

3. Our continuous failure to obtain a culture in the Löwenstein-Jensen medium with bacilli developed in the hamster from borderline precursors, in contrast with the culture we did obtain from bacilli developed in the hamster from precursors of lepromatous origin.

4. The capacity of an antigen prepared from our strain of bacilli, developed in the hamster from borderline precursors, to sensitize to human lepromin in lepromatous patients.

5. The similar positivization observed with an antigen from the Binford strain of the bacillus from hamster lesions of lepromatous origin and the capacity of an antigen from Binford's cultivable strain of lepromatous origin to do the same.

6. Finally, the incapacity of our hamster antigen of borderline origin to induce a positive Mitsuda reaction after the lipid constituents had been removed with chloroform.

From the latter observation it seems highly probable that the specificity of the bacillary antigen depends on one or more lipid components. It is not clear, however, whether the haptenic lipids in question pertain exclusively to the bacillus or to the tissue or to both. In the Dharmendra method of separating the bacilli the chloroform would remove the lipids from tissue particles as well as from the bacilli.

There is room here for an investigation of the haptenic properties of lipids from the bacilli as well as from human skin tissue of healthy positive reactors. It is also necessary to investigate the molecular structures that determine the specificity of lipid haptenes.

The capacity to synthesize a specific hapten is undoubtedly genetic in nature. We are faced here with a problem that involves both human and bacterial genetics.

It has been known for many years that acid-fast bacilli closely resembling saprophytic mycobacteria can be cultivated from precursors in lepromatous lesions. Many strains of such bacilli are currently cultivated in the American Type Culture Collection in Washington, D.C. These forms can be considered taxonomically from two points of view: (1) They may be "wild" mycobacterial forms that have been introduced into the lesions as a secondary infection and found the intracellular medium highly favorable for proliferation in the presence

of "true" *M. leprae*, or (2) they may be mutants that have evolved from true uncultivable strains of *M. leprae*.

In the light of modern bacterial genetics it would seem entirely possible that two strains of a bacterial species in a general population would be uncultivable, each from a different genetic deficiency, and yet that one of them, acting as donor, could supply the other with the genetic factor needed to synthesize an enzyme necessary for the utilization of a given culture medium.

The first postulate would appeal to many leprologists who still like to think of *M. leprae* as a taxonomic entity existing as an obligatory intracellular parasite. Others might find it rather improbable that a "wild" and practically ubiquitous mycobacterial species would be so thoroughly adapted to the intracellular environment in the presence of the "true" *M. leprae*.

The second postulate would seem to be the more probable in the light of bacterial genetics. If we accept it, we are bound to believe that the element of chance has had more to do with successful cultures and positive inoculations in animals than ingenious technical design.

Through interchange of genetic material between bacterial cells contiguous by chance, many mutations may be possible. Some of these would be lethal and some viable in culture through increased autonomy. Among the latter some mutant of unusual properties may again result. That is probably the case with the bacillus of the Binford culture that gave an antigen capable of sensitizing lepromatous patients to human lepromin.

It would be well for leprologists in the future to leave no cultivable mutant of *M. leprae* unstudied with respect to its immunologic properties. It should be the task of biochemists to investigate the molecular structure at the root of immunologic specificity in leprosy and of geneticists to investigate the induction of the natural synthesis of such structures. Before these functions have been made clear, we may expect that mere trial and error will give us an immunologic therapy for use at least in the early stages of lepromatous leprosy.

#### SUMMARY

Leprosy has been transmitted to hamsters by inoculating them with bacilli from borderline lesions in man. After an incubation period of eight months, which was reduced to four in later passages, nodular lesions with a great many acid-fast bacilli were present at the site of the inoculation.

The investigators believe that a new variant of *M. leprae* has been produced by mutation. This concept is supported by the observation under the electron microscope of differences between the human and hamster lesions and their bacilli and by differences in the immunologic properties of the human and the hamster strains.

An antigen prepared by the trypsinization method from the bacilli

of the hamster lesions gave a positive skin reaction in 42 lepromatous patients, in contrast to what is persistently observed with human lepromin. However, when the latter was administered on the opposite forearm of the patient simultaneously with the hamster antigen, or even a month later, a positive reaction was evoked. This induced capacity of the patient to react positively to human lepromin was still observed a year after the administration of the hamster antigen.

Two antigens prepared by C. H. Binford, one from a cultivated strain of *M. leprae* and the other from a strain developed in the hamster, were shown by the investigators to have a similar capacity to evoke a positive Mitsuda reaction.

Investigations will be continued with special reference to the molecular structure of the immunizing factor, which may be a lipid, and the induction of the biologic capacity to produce it.

The possibility is foreseen of developing an immunologic therapy, on the basis of mutants of *M. leprae*, for use at least in the early stages of lepromatous leprosy.

#### RESUMEN

Ha sido transmitida la lepra al hamster, por inoculación de bacilos provenientes de lesiones borderline del hombre. Después de un período de incubación de 8 meses, que se redujo a cuatro en los pases avanzados, aparecieron lesiones nodulares en los sitios de inoculación, que contenían gran cantidad de bacilos ácido-resistentes.

Los autores creen que una nueva variante del *M. leprae* ha sido producida por mutación. Este concepto es reforzado por los hechos observados al estudio con el microscopio electrónico, observándose diferencias entre la lesión humana y la del hamster así como del bacilo en dichas lesiones, así como diferencias en las propiedades inmunológicas entre la cepa humana y la del hamster.

Un antígeno preparado por el método de tripsina de bacilos provenientes de una lesión del hamster dió una reacción cutánea positiva en 42 enfermos lepromatosos en contraste de lo que se observa con lepromina humana. Sin embargo cuando se aplicó el test con lepromina humana en el antebrazo opuesto simultáneamente con la aplicación de antígeno del hamster, o aún un mes después se provocó una reacción positiva. Esta capacidad del paciente de reaccionar positivamente a la aplicación de lepromina humana fué observada hasta un año más tarde después de la aplicación del antígeno de hamster.

Dos antígenos preparados por C. H. Binford, uno proveniente de una cepa cultivable y la otra proveniente de una lesión del hamster hicieron positiva la reacción a la lepromina en enfermos lepromatosos.

Se continuarán las investigaciones a fin de estudiar la estructura molecular del factor inmunizante, que puede ser un lípido, así como la inducción de la capacidad biológica para producirlo. Se considera la posibilidad de desarrollar una terapia inmunológica teniendo como base los mutantes del *M. leprae*, para ser usados como mínimo en las etapas iniciales de la lepra lepromatosa.

#### RESUMÉ

On a réussi à transmettre la lèpre au hamster par inoculation avec des bacilles des lésions borderline humaines. Après une période d'incubation de 8 mois, réduite dans les passages ultérieurs à 2, on a observé à l'endroit de l'inoculation des lésions nodulaires avec une grande abondance de bacilles acido-résistants.

Les investigateurs eroient qu'un nouveau type de *M. leprae* a été développé dans le hamster par mutation. Cette idée était justifiée par les observations faites au microscope électronique qui ont révélé des différences entre les lésions humaines et leurs



bacilles et celles du hamster et leurs bacilles, ainsi que par les différences entre les propriétés immunologiques des deux types.

Un antigène préparé avec des bacilles du hamster par la méthode de trypsination a donné une réaction positive chez 42 malades lépromateux, contrairement à ce qu'on observe toujours en usant la lépromine humaine. Néanmoins, en injectant cet antigène à l'autre avant-bras du malade simultanément avec l'antigène du hamster ou même un mois après, les résultats obtenus étaient positifs.

Cette capacité induite dans les malades de réagir positivement à la lépromine humaine se manifestait encore une année après qu'ils avaient reçu l'injection d'antigène de hamster.

On a prouvé que deux antigènes préparés par C. H. Binford, l'un fait avec un type cultivé de *M. leprae* et l'autre d'une variation développée dans le hamster, ont la même capacité d'évoquer une réaction Mitsuda positive.

Les investigateurs continueront leurs expériences afin de déterminer la structure moléculaire du facteur immunisant—une lipéide peut-être—ainsi que le mode de l'induction de sa synthèse biologique. Ils envisagent le développement d'une thérapie immunologique, basée sur les propriétés de nouvelles variations du bacille et applicable au moins dans les cas lépromateux pas trop avancés.

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