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CULTIVATION OF B. LEPRAE WITH EXPERIMENTAL LESIONS IN MONKEYS[1]

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Leprosy remains one of the infectious diseases of world wide importance in which the etiologic agent is surrounded with more or less obscurity. Since the discovery by Hansen in 1872 of small rods lying within the "lepra cells" this organism has been generally accepted as the cause of the affection, and yet of the many reports on the cultivation of Hansen's bacillus obtained from typical lesions of the disease there is none that has been accepted as establishing proof of the actual cultivation *in vitro* of *B. leprae.* This has been due in part to the wide variety of microörganisms which have been cultivated, the failure to rule out contaminations in the handling of leprous material for cultivation purposes as well as decisive experimental proof of the pathogenesis of any of the various organisms isolated. No less than seventeen investigators have described the cultivation of diphtheroids from leprosy-material (see table 1); another five or six have reported the cultivation of acid-fast chromogenic organisms with about the same number having described nonchromogenic cultures of acid-fast bacilli from leprosy lesions. There have, in addition, been a small group of investigators who have cultivated anaërobic bacilli from lesions of the disease. In the face of so many diverse reports it is not at all surprising that the question of the etiologic relationship of any of these organisms to leprosy should be regarded with suspicion. A similar situation exists with regard to the production of experimental lesions of the disease in laboratory animals. Several investigators have described experimental infection in such animals as rabbits, guinea pigs, rats, mice, monkeys, etc. (see table 2), with infected tissues from lepers and a

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TABLE 1—*Summary of positive culture experiments with leprosy material*

Author	Date	Material	Medium	Animal Inoculated	Results
			Diphtheroids		
Neisser	1886	Leprosy, post mortem tissue	Blood-serum-egg		
Bordoni-Uffreduzi	1889	Leprosy, post mortem tissue	Glycerin-blood-serum. Subculture on glycerin agar	Guinea pigs Rabbits Mice	Negative Negative Negative
Babes	1889	Leprosy, post mortem tissue	Glycerin-human blood-serum		
Gianturco	1889	Leprosy (nodule)	Glycerin-human serum agar		
Lévy	1897	Leprosy (nodule)	Glycerin-agar-human blood	Guinea pigs Rabbits Mice	Negative Negative Negative
Sponck	1898	Leprosy (nodule)	Glycerin-potato. Human blood serum agar		
Czaplewsky	1898	Leprosy (nose and nodules)	Glycerin-sheep blood serum	Guinea pigs Rabbits Mice	Negative Negative Negative
Teigh	1899	Leprosy (nasal discharge			
Kedrowsky	1901	Leprosy (nodule)	Placenta agar. Glycerin agar	Rabbits	Granulomas containing acid-fast bacilli 8 months later

TABLE 1—*Continued*

Kliten	1905	Leprosy (nodule)		Guinea pigs, rabbits	Lesions and recovery of granular organisms
Shiga	1909	Leprosy	Glycerin-serum potato		
Bayon	1911	Leprosy	Placenta extract agar. Horse serum with 2 per cent ground B, Smegma. Dorset's egg	Mice	Recovered organisms from glands
Williams	1911	Leprosy (nodules)	Lemco broth. Dorset's egg		
Reenstierna	1913	Leprosy		*M. rhesus*	Lesions
Duval	1918	Leprosy		Laboratory animals	Negative
Wolbach and Honeij	1914	Leprosy	Ascitic fluid Dextrose agar		
Walker	1922	Leprosy (nodules)	Glycerin veal broth agar. Various mediums		
			Chromogenic acid fasts		
Rost	1905	Leprosy	Salt-free broth		
Williams	1911	Leprosy	Dorset egg		
Clegg	1909	Leprosy (nodules)	Symbiosis with *V. cholera* and amoebae. Glycerin agar	Guinea pigs	Negative
				White rats	Negative
				Monkeys	Negative
Duval	1910	Leprosy (nodules)	Various: Clegg media, banana agar, etc.	Guinea pigs	Negative
				Rabbits	Negative
				Rats	Negative
				Japanese dancing mice	Lesions
				M. rhesus	Lesions
Walker	1923	Previous diphtheroid cultures. Lepers	Clegg's medium		
Watanabe, Harasawa and Ono	1931	Leprosy (nodules)	Egg yolk agar	Japanese monkeys	Negative
				Guinea pigs	Negative
				Mice	Negative

TABLE 1—*Concluded*

Author	Date	Material	Medium	Animal Inoculated	Results
Non-chromogenic acid fasts					
Weil	1905	Leprosy	Various: egg, veal broth sea-water glycerin, peptone and glucose broth		
Karlinsky	1912	Leprosy (nodules)	Vesicular fluid	Guinea pigs	Negative
Marchoux	1911	Leprosy (nasal discharge)	Potato glycerin ascitic agar	Rats	Recovered organisms
Twort	1910	Leprosy (nasal discharge)	Dorset's egg-glycerin and ground *B. tuberculosis*		
Duval and Wellman	1912	Leprosy (nodules)	As above	Rabbits	Negative
Wherry	1930	Leprosy	Glycerin agar with egg. Under gaseous tension		
Anaerobic organisms					
Ducrey	1892	Leprosy (nodules and blood)	Campana medium. Agar-grape sugar		
Campana	1894	Leprosy	Same as above		
Serra	1910	Leprosy (nodules)	Same as above	Guinea pigs	Negative
				Rabbits	Negative
				Dogs	Negative
				Rats	Negative
Actinomyces					
Kedrowsky	1901	Leprosy (nodules)	Placenta agar. Glycerin agar	Rabbits	Granulomas
Walker	1929	Leprosy (also soil)	Various		

few workers have reported the production of experimental lesions with some of the various cultures referred to above. None of these have been accepted as authentic lesions of leprosy. It is quite apparent then that the postulates of Koch, upon which for nearly half of a century we have based evidence of specific etiology in infectious diseases, have not been satisfactorily or acceptably fulfilled for the etiologic agent of leprosy. However, it can be stated without fear of serious contradiction, that most authorities regard *B. leprae* of Hansen as the probable cause of this malady.

While so little is known about the specific microörganism causing leprosy the disease has received great attention with respect to many of its other phases. Notable reviews and surveys of the subject have been published by Rogers and Muir (1), Zambaco (2), de Souza-Araujo (3), etc. In addition various texts on tropical diseases include summaries of what is known regarding the disease and many scientific periodicals also contain reviews and original papers dealing with this subject. Rogers (4) places the number of cases of leprosy in the world at about three million while Heiser estimates the number of cases at four million. Even should the exact number, if known, surpass one million the disease would still constitute a serious public health and social problem in many parts of the world. The study of leprosy has been complicated by its chronicity. The bacillus of Hansen most probably possesses a very low virulence and many other factors, such as hot and humid climate, nutrition, lowered general resistance due to allied diseases and infections, age, race, etc. are thought to predispose to human infection. Much has been learned regarding the clinical aspects of leprosy and two general types of the disease, the nodular and nerve forms, are recognized. Various modifications of these types occur and the clinical manifestations vary considerably. The disease is known not to be hereditary. Rodríguez (5) has found no evidence of hereditary infection in children at the Culion leper colony and no hereditary predisposition has as yet been determined. The incubation period of the disease is unknown. Rogers, however, places the incubation period in 80 per cent of infections at not more than five years with an average incubation period of two years and eight months for the groups studied by him. Some leprologists hold that it is absurd to speak of an incubation period in this disease, because of its slow development, in the sense this phrase is usually employed in other infec-

TABLE 2—*Experimental leprosy in animals*

Author	Material Inoculated	Result
	Guinea pigs	
Klitten	Cultures of diphtheroids isolated from leprosy nodules	Lesions with recovery of short granular organisms
Clegg	Cultures of chromogenic acid-fast organisms isolated from leprosy nodules	Lesions
Iwanow	Leprosy material	Lesions
Kikuchi	Leprosy material	Lesions
Sugai	Leprosy material	Lesions
de Souza-Araujo	Leprosy material	In one animal evidence of multiplication of bacilli
	Rabbits	
Kedrowsky	Cultures of diphtheroids isolated from leprosy nodule	Cultures recovered from rabbits 8 months following inoculation
Klitten	Cultures of diphtheroids isolated from leprosy nodule	Lesions with recovery of granular forms
Damsch, Melcher, Ortmann, Vossius and Baranikow	Leprosy material	Lesions
	Rats	
Marchoux	Nasal secretion from leper	Subcutaneous abscesses containing acid-fast bacilli
Shiga	Leprous tissue and cultures	Lesions
de Souza-Araujo	Leprosy nodule	Mesenteric nodules. Tumor and abscess
	Mice	
Duval	Cultures of acid-fast chromogenic organisms from leprous tissue	Lesions in Japanese dancing mice
Sugai	Leprosy material	Abdominal nodules—free or adherent to viscera. In Japanese dancing mice
Bayon	Cultures of diphtheroids from nodules	Recovered acid-fast bacilli from glands which were later subcultured
de Souza-Araujo	Fresh lepromata	Nodules; tumors; abscesses

TABLE 2—*Concluded*

Author	Material Inoculated	Result
	Monkeys	
Nicolle	Leprosy nodule into two bonnet monkeys	Nodular lesions, 62 days
Nicolle, Marchoux and Bourret	Leprosy tissue into chimpanzees	Negative or doubtful
Kryle	Leprosy tissue into three rhesus monkeys	Nodular lesions, 18 to 22 days
Duval and Duval and Couret	Cultures chromogenic acid-fast organisms from leprosy material into 2 rhesus monkeys	Disseminated lesions
Clegg	Cultures	Negative
Reenstierna	Leprosy tissue into a *M. sinicus* and *M. rhesus*. Later 6 other monkeys	Nodular lesions, 39 days
Roffo	Leprosy tissue	Lesions
Franchini	Leprosy tissue	Lesions
Schöbl, Pineda and Miyao	Leprosy tissue into two Philippine monkeys. Repeated inoculation	Nodular lesions
de Souza-Araujo	Leprosy tissue emulsion into *Pseudocebus* and *M. rhesus*	Nodular lesions, 23 to 62 days

tious processes. The nodular type of the disease seems to possess greater infectivity than the nerve forms. The stages of infection have been described by Muir as three, in the first of which there is a local multiplication of bacilli, in the second an inflammatory reaction in which general toxemia may appear, and a third phase of resolution marking the end of the reactionary stage. One or all of these stages may exist at one time in a given patient and either a part or the whole of the lesion may be involved. Rogers points out that early lesions of leprosy are quite characteristic and may frequently be diagnosed by those not trained in the art of medicine. Rodríguez in the Philippines has reported that in children at Culion a single primary lesion was the first manifestation of the disease in no less than 85 per cent while the remaining 15 per sent showed a generalized rash or multiple macules appearing simultaneously.

Children exhibit a high degree of susceptibility if left in contact with leprous parents, the percentage running around 33 to 40 per cent at Culion if suspicious infections are also included.

In any study of the cultivation of *B. leprae* and attempts to produce experimental lesions of leprosy in animals the clinical aspects of the disease are important. In the first place it would seem that such experiments would be most likely to succeed if early nodular lesions were employed as experimental material. In these one would expect to find the greatest numbers of multiplying bacilli. Since, in the vast majority of cases, the primary lesions are single lesions attempts should be made to reproduce these in the experimental animal. Since younger individuals (children and young adults) are apparently more susceptible to infection it would appear that young animals might in the end be expected to yield the highest percentage of positive infections. Prolonged incubation periods should not in themselves prove discouraging. In planning the protocols of the experiments described in this paper we have endeavored to keep these various factors in mind as fundamental to the problems under study.

There have been many reports in the literature dealing with the experimental transmission of leprosy to animals. These reports have been encouraging but not conclusive. Experimental transmission of the disease in man, however, has been very discouraging and there is perhaps no case of experimental human transmission of the disease on record (out of 145 human inoculations) which can be accepted as a proven fact (with the possible exception of the Arning case in Honolulu). Since man appears to be the natural host for leprosy the reason for his non-susceptibility to experimental infection is little understood. Many explanations have been suggested. That leprosy is communicable from man to man is generally accepted particularly when one reviews, as Rogers and Muir point out, the outbreak of the disease on Rodríguez Island where in the course of forty or fifty years 23 cases of the disease developed from the spread of the infection from one original case. These authors also mention the introduction of leprosy into Natal by two infected persons which eventually led to over 200 cases of the disease. Other similar observations have been recorded. In general the failure to induce experimental lesions in human subjects must be due either to resistance of the host or to a low virulence of the leprosy bacillus, or to both. What factors make up host resistance are not known except in a

very general way and these have already been mentioned under predisposing causes. The disease in man is an extremely slow process which indicates the ability of the host to withstand the infection for a considerable period during which the virulence of Hansen's bacillus may be modified even though, in the end, the patient succumbs to infection. In other words, the leprosy materials usually employed to produce experimental infection in either man or experimental animals may contain organisms of such modified or low virulence that they are unable to combat the resistance of the new host in which they are implanted. Such an explanation is at least worth considering as a working hypothesis.

Leprosy does not occur as a natural infection in any animal except possibly the rat. Rat leprosy and human leprosy are, however, not identical but merely similar infections. Human leprosy cannot be reproduced in rats with any certainty although investigators have reported suspicious lesions in rats following inoculation with human leprous material. Marchoux (6) has described a strain of rat leprosy organisms which, when fresh, infected a normal animal but when kept outside the body for twenty-four hours it apparently lost its virulence. Such an observation is important for it suggests a possible explanation for the many failures to cultivate the human leprosy bacillus outside the body and to produce experimental lesions with human material containing *B. leprae.*

In the experiments which are here described we have attempted to divide the work into three parts, i.e., (*a*) to attempt to transfer the disease to monkeys with human leprosy material; (*b*) to cultivate the acid-fast organism (presumed to be Hansen's bacillus of leprosy) present in human leprosy lesions; and (*c*) with cultures of this organism to reproduce experimentally the lesions of leprosy in monkeys.

EXPERIMENTAL LESIONS IN MONKEYS WITH HUMAN LEPROSY MATERIAL

A review of the literature (see table 2) indicates that a considerable number of investigators have attempted to produce leprosy experimentally with human leprosy material. Monkeys, chimpanzees, rabbits, guinea pigs, rats and mice have all been utilized for this purpose. No attempt will be made to review in detail all of the literature bearing upon this phase of the problem but we will

mention briefly some of the outstanding work and reported results. Nicolle has described experimental nodular lesions in two bonnet monkeys which were inoculated with human leprosy material. The nodules appeared in sixty-two days. Nicolle, Marchoux and Bourret obtained negative results with human leprosy material inoculated into chimpanzees. Kryle produced nodular lesions in three rhesus monkeys with human leprosy material, the nodules appearing in eighteen to twenty-two days. Clegg injected monkeys with cultures with negative results. Bradley has reported the production of nodular lesions in monkeys which appeared within ten weeks following inoculation. Roffo, and also Duval and Duval and Couret, have reported experimental leprosy lesions in monkeys. More recently Reenstierna (7), Franchini (8) and Schöbl, Pineda and Miyao (9) have described experimental leprosy lesions in monkeys following the injection of human leprosy material. It appears, then, that many investigators have been able to induce lesions suggestive of leprosy in monkeys of different species utilizing as a source of material the infected tissue from human cases of leprosy. Other animals have also been employed. Thus Clegg, as well as Kliten, has reported lesions in guinea pigs produced with cultures obtained from human leprosy cases. Other investigators employing various cultures, thought to be cultures of *B. leprae,* have produced lesions in mice (Duval, Bayon); in rats (Marchoux, Shiga); and in rabbits (Kedrowski, Kliten). Damsch, Melcher, Ortmann, Vossius and Barani-kow have also reported lesions in rabbits produced with human leprosy material and recently de Souza-Araujo (10) has described lesions in both white mice, rats, guinea pigs, and in monkeys with leprosy tissue from human cases.

It seems quite possible from the reports of the various investigators mentioned that lesions suggestive of leprosy may be produced in experimental animals, not only with leprosy material taken direct from leprosy patients but also with cultures of organisms which have been isolated from human leprosy material. Still there has been considerable skepticism in the minds of many that leprosy lesions have actually been reproduced in animals or that Hansen's bacillus has been cultivated on artificial mediums. There has been no clear-cut story of the infection of experimental animals, such as monkeys, with human leprosy material, the isolation and cultivation of *B. leprae* followed again by the experimental production of lesions in the

experimental animal with such cultures. A review of the literature, however, seems quite convincing that suggestive leprosy lesions have at least been produced experimentally in monkeys with human material. Reported work with cultures in other animals is not so convincing and becomes less so when one considers the large number of different organisms which have been cultivated from human lesions of this disease and with which such experimental work has been performed.

EXPERIMENTAL

In the leper colony of the Insular Department of Health of Porto Rico there are 60 cases of leprosy, both male and female, and it is estimated that there exist perhaps no more than forty other lepers on the Island who are not under treatment. From the male patients at the leprosarium we selected a group of five showing marked early nodular lesions. All previous experiments which we carried on to those reported in this paper, which were designed to induce lesions of the disease in monkeys and other laboratory animals had failed. Monkeys, rabbits, and guinea pigs have, in the past, been inoculated by us with emulsified leprosy nodules by several methods (intratesticularly, intraperitoneally, intramuscularly, intracerebrally, etc.) and, with one exception, the animals have remained in good health and have showed no evidences of the disease. One monkey became ill and died a year following intratesticular inoculation with leprosy material and on autopsy was found to have succumbed to generalized tuberculosis. The work of recent investigators, however, indicated that experimental lesions may be produced in monkeys following intradermal inoculation of leprosy material. We therefore decided to utilize the intradermal route of infection and by this method we have succeeded in producing granulomatous lesions which are suggestive of early leprosy, in both *Macacus rheus* and *Cebus olivaceous* monkeys.

The lepers were brought to the University Hospital of the School of Tropical Medicine and typical nodules from the arms and ears were removed under rigid aseptic technique by Dr. Wm. R. Torgerson of the surgical staff. These nodules were dissected carefully from the underlying tissues and removed without skin. In the laboratory they were ground up finely with glass rods in sterile test glasses and emulsified in physiological saline solution. The various emulsions were examined by stained preparations for acid-fast or-

ganisms and were found to be rich in acid-fast bacilli, presumably *B. leprae* Hansen. Cultures were then prepared with the leprosy nodule emulsions on various mediums which will be described later. The emulsions were then pooled and eight young *Macacus rhesus* and five young *Cebus olivaceous* monkeys were inoculated intradermally over the eyebrow with 0.25 cc. of the material. (See table 3.)

It will be noted in table 3 that seven of the eight *Macacus rhesus* and all five of the Cebus monkeys developed nodular lesions at the site of inoculation in from eighteen to twenty days following injection of the leprosy material. These nodules were firm, hard, red and showed no tendency to soften. However, within a week follow-

TABLE 3—*Inoculation of monkeys with human leprosy material*

Monkey Number	Inoculated Intradermally with Human Leprosy Nodule Emulsion	Result
	cc.	
M-1	0.25	Developed nodule in 18 days
M-2	0.25	Negative
M-3	0.25	Developed nodule in 19 days
M-4	0.25	Developed nodule in 18 days
M-5	0.25	Developed nodule in 18 days
M-6	0.25	Developed nodule in 20 days
M-7	0.25	Developed nodule in 18 days
M-8	0.25	Developed nodule in 18 days
C-1	0.25	Developed nodule in 19 days
C-2	0.25	Developed nodule in 20 days
C-3	0.25	Developed nodule in 20 days
C-4	0.25	Developed nodule in 19 days
C-5	0.25	Developed nodule in 18 days

ing the appearance of the nodules one of the nodules ulcerated and another was doubtful because it was thought the animal might have injured himself at the site of inoculation in moving about in the cage. From the latter some serum was expressed from the lesion and acid-fast bacilli were demonstrated in the stained smear. The ulcerated nodule was removed for histologic study as were several of the other most typical nodules. Parts of the nodules were emulsified and cultures and stained smears were made with this material. The smears showed numerous acid-fast bacilli. The appearance of such lesions in monkeys following the inoculation with leprosy material from human cases of the disease is in accordance with the work

reported by several other investigators who have reported incubation periods ranging from eighteen days to seventy days. The shortest incubation period in monkeys which corresponds with the experiments described in this paper has been reported by Kryle and by the Souza-Araujo while the longest are those reported by Nicolle of sixty-two days and of Bradley who reports an incubation period of ten weeks.

Histological examination of sections of three of the most typical nodules kindly examined by Dr. William C. von Glahn revealed definite granulomas consisting of nodular accumulations of cells of the large mononuclear type with infiltrations of lymphocytes and clumps of polymorphonuclears. The characteristic foamy cellular cytoplasm was not present in these early lesions and the first acid-fast stains of these sections revealed no bacilli although subsequent study of some of these sections with a modified technique showed a few acid-fast organisms and acid-fast granules to be present. Multinucleated giant cells were met with in some of the sections. The lesions were distinctly very early and histologic pictures presented are very suggestive of early lesions of leprosy which we believe they may be. However, such lesions in monkeys, in our experience, vary greatly in size ranging from 0.5 to 2 cm. in diameter, and increasing gradually during the first ten days or two weeks following their appearance. They then tend to regress and disappear entirely within another three or four weeks. Schöbl, Pineda and Miyao have reported the production of nodular lesions in the Philippine monkey by repeated intradermal inoculation of human leprosy material and they believe there is an allergic factor involved in the production of such lesions. Our experiments would not indicate this since, in the majority of monkeys of the two species we have used, nodular lesions are produced following a single injection of the leprosy material. This also coincides with the experiments reported by Reenstierna who has described experimental lesions in monkeys practically identical with our own.

In view of the several reports of other investigators concerning the experimental production of granulomatous lesions in monkeys with human leprosy material and the positive results we have obtained in our experiments it seems most likely that in these lesions we have fairly definite evidence of experimental transmission of the infection to this experimental animal. The monkey, however, ap-

parently possesses considerable natural immunity to this infection for such experimental lesions are not progressive and usually the infection is aborted within a few weeks following the appearance of nodular lesions. Control animals have been inoculated with media and various other substances (wax, ground guinea pig lymph gland, etc.) and no lesions have apparently resulted from such inoculations.

One of the most typical nodules produced in this series on a *Macacus rhesus* was removed for histologic study and part of the nodule was emulsified for the purpose of attempting to pass the infection in series in other monkeys. The emulsion contained acid-fast bacilli but they were not numerous. Three normal *Macacus rhesus* were inoculated intradermally over the eyebrow with 0.25 cc. of this emulsion but none of these animals developed lesions. We are at a loss to account for the resistance manifested by these animals but we should point out that the emulsion did not contain the massive infection which was given to the first series of animals in direct inoculation from man and it is apparent that the new host was able to resist the implantation of the amount of infection which was given. These three animals do, however, illustrate that the mere injection of ground nodular tissue (even when containing a few acid-fast bacilli) is not followed by the appearance of the typical nodules which we have described as a result of inoculation with human leprosy material. They may therefore be regarded as additional controls. Other investigators have also reported failure to pass on such experimental lesions in series.

CULTIVATION OF B. LEPRAE

Leprosy has been the subject of extensive bacteriological investigation during the six decades that have elapsed since the observations of Hansen. The bacilli noted by this worker to be constantly present in the cells of freshly excised lepromata are universally believed to be the specific cause of this malady. Notwithstanding the exhaustive attempts to cultivate *B. leprae* and the periodic reports of success, it appears to be still the fact that the organism has not been artificially cultivated *in vitro*. An understanding of the disease would be greatly aided by the cultivation of the leprosy bacillus and a pure antigen would materially assist in the related serological studies of susceptibility and the production of immunity.

A review of the literature (11-16) shows that a variety of organisms have been cultured from leprosy material. On classification

it will be seen that they fall quite naturally into three groups: (*a*) non-acid-fast diphtheroids; (*b*) chromogenic acid-fast bacilli; and (*c*) non-chromogenic acid-fast bacilli. What relation, if any, these organisms bear to leprosy is a question. Are they contaminating organisms that have nothing to do with the disease; or are they germs in some way associated with the true causative agent which has not as yet been cultivated, such a relationship, for example, as exists between the hog cholera bacillus and hog cholera; or are they different stages in a life cycle of the leprosy bacillus, the acid-fast phase of which is common to diseased tissue? All three possibilities have had their sponsors and suggestive experimental proof.

It is of interest, however, that no one organism of the above-mentioned group has been cultivated with any degree of regularity from sources known to be rich in Hansen's bacillus even when the exacting requirements of the most fastidious germs were complied with. To give, by way of example, the results of one investigator: Walker reported 2,363 attempts using over 50 different mediums, aerobic and anaerobic environments, variations in oxygen and CO_2 tension, variation in hydrogen-ion concentration, temperature, etc. with a net result that 13 coccoid, 66 dipththeroid, and 1 actinomyces strains were isolated.

If, as has been suggested, Hansen's bacillus is only the tissue-invading stage in the life cycle of a pleomorphic organism it is without precedent in bacteriological work that such a stage which is apparently very stable in the tissue and on the mucous membranes of the nose cannot be isolated and maintained under artificial conditions with such attributes.

Only a few (17) of the many investigators have considered the gaseous environment when attempting to culture the leprosy bacillus artificially and yet it is reasonable to believe from the recent literature[2] on the gaseous metabolism of bacteria that consideration of the respiratory requirements may play a very important rôle in obtaining primary isolations. With this consideration in mind the experiments to be described were undertaken.

MATERIALS AND METHODS

Ten mediums, recognized as having merit for the cultivation of acid-fast organisms and that have been used at various times in attempts to isolate *B. leprae* were prepared as follows, and placed

[2] See Soule, p. 250-67. The Newer Knowledge of Bacteriology and Immunology, edited by E. O. Jordan and J. S. Falk, Chicago, 1928.

in 18 by 150 mm. culture tubes. Witte's prewar peptone, 1 per cent, and Kahlbaum's sodium chloride, 0.5 per cent, were incorporated in all of the broth.

Glycerol potato and glycerol broth potato. Potatoes from a number of different sources were selected and the regular slants made. A few drops of glycerol (*K*) were poured over the potato surface, in the preparation of the former medium, and 2 cc. of 5 per cent glycerol broth were added to the slants in the latter medium, previous to sterilization.

Petroff's egg and Dorset's egg mediums. The two mediums were prepared according to the standard methods.

Hormone glycerol agar. The broth was prepared by the infusion of fresh beef, decantation was resorted to rather than filtration at all stages in the process and glass vessels were used throughout, 1.5 per cent agar and 5 per cent glycerol were added previous to sterilization.

Ordinary glycerol agar. This was the usual 5 per cent glycerol, 2 per cent agar infusion medium.

Rabbit blood agar and rabbit serum agar. Equal volumes of the melted hormone glycerol agar at 50° C. and sterile rabbit's blood or rabbit's blood serum were mixed and cooled in the slanting position.

Glucose brain broth. The usual medium of Rosenow.

Koori Konnyaku. Strips of this carbohydrate were moistened with hormone broth. The physical state of this substance was not altered by subsequent sterilization.

EXPERIMENTAL

Patients with nodular leprosy were available. Well isolated nodules located on the arms and ears were selected and the skin over the areas was thoroughly washed with soap and water previous to several applications of tincture of iodine. A local anaesthetic was administered and 4 nodules from 3 different patients (fig. 13) were enucleated with aseptic technique. The tissue was emulsified with physiological saline and filtered through sterile glass wool, after a microscopic examination of the filtrate for the presence of the acid-fast organisms, the emulsions were rich in these forms, two drops were transferred to each tube of medium with sterile bulb pipettes. Subsequently, the cotton plugs were flamed and pushed within the tubes.

When cool, the inoculated tubes were divided into 4 series and placed in Novy jars of 2,400 cc. capacity so that at least 2 inocu-

lated tubes of each of the 10 mediums, of each of the nodular series were exposed to the various environments. The jars were closed as usual, attached to a vacuometer and the desired gaseous atmospheres were introduced by the procedure of Novy, Roehm and Soule (18).

In this manner freshly inoculated tubes were placed in atmospheres containing 10 per cent O_2 and 10 per cent CO_2; 20 per cent O_2 (air) and 10 per cent CO_2; 40 per cent O_2 and 10 per cent CO_2; 0.0 per cent O_2 and 10 per cent CO_2; plus air controls. The early observations of Wherry and Ervin (19) that CO_2 is essential to the growth of the tubercle bacillus and the recent work of Novy and Soule (20), wherein, it is emphasized that a definite concentration of free CO_2 is absolutely necessary to maintain the physicochemical equilibrium between the extra- and intracellular CO_2 directed the use of free CO_2 in these attempted isolation experiments of the leprosy bacillus. From previous work with bacteria and protozoa a concentration of 10 per cent CO_2 seemed to be the most favorable for the primary isolation of organisms, therefore, this tension was introduced in all of the jars in the present series of experiments and the O_2 concentration was varied.

The CO_2 was prepared in a Kipp generator from the interaction of marble and dilute HCl. The gas was washed previous to its introduction into the jars by passage through a saturated solution of Na_2CO_3. The oxygen was obtained in the small tanks as used for medical purposes. Hydrogen was made in a Kipp generator from purified Zn and dilute HCl and washed by passage through solutions of $AgNO_3$, Na_2CO_3, $Pb(C_2H_3O_2)_2$ and alkaline pyrogallate.

To obtain the desired tensions of the different gases the jars were attached to the vacuometer and after exhaustion to the desired negative pressures the calculated number of millimeter of the gases were run in. No burette analyses were made but previous experience with this method for producing gaseous mixtures of definite composition indicated that the tensions desired were obtained with a variation of less than 0.5 of 1 per cent.

After the adjustment of the gaseous tensions the jars were placed in incubators at 37.5°C. The ordinary air controls subsequent to the flaming of the tubes were wax sealed and a tiny hole was put through the wax with a hot wire to insure an interchange of air as suggested by Novy and Soule (21) in their studies on the respiration of the tubercle bacillus. These tubes were then incubated at 37.5°C. in close proximity to the jars.

The air controls were carefully observed for evidence of growth and contamination. The jars were regularly removed from the incubators and inspected but they were not opened during these routine examinations. At the end of four weeks of incubation the jars were opened and several contaminated tubes, a spore forming aerobe, were discarded. There was no macroscopic evidence of growth in the uncontaminated tubes. Several were therefore selected and smears were prepared from material obtained from the surface of the medium and stained by the Ziehl Nielson method. Microscopic

TABLE 4—*A comparison of the growth obtained on the mediums used for the isolation of B. leprae in various concentrations of O_2 and CO_2 in Novy jars incubated at 37°C. for 6 weeks*—Patient 1*

Mediums	Percentage of CO_2 and O_2				Air Controls
	O_2, 10.0; CO_2, 10.0	O_2, 20.96; CO_2, 10.0	O_2, 40.0; CO_2, 10.0	O_2, 0.0; CO_2, 10.0	
Glycerol potato	?†	+	+	0	0
Glycerol broth potato	?	+	+	0	0
Petroff's egg	?	+	+	0	0
Dorset's egg	0	0	+	0	0
Hormone glycerol agar	+	+	+	0	0
Ordinary glycerol agar	0	0	0	0	0
Rabbit blood agar	0	0	0	0	0
Rabbit serum agar	0	0	+	0	0
Glucose brain broth	0	0	0	0	0
Koori Konnyaku	0	0	0	0	0

* Those tubes showing positive growth of acid-fast rods were subcultured to same mediums and returned to previous gaseous environments.

† + indicates tiny colonies with well formed rods; ? indicates well formed rods with question of colonies; 0 indicates highly granular degenerating rods.

examination indicated proliferation in several tubes, therefore the tubes were returned to the jars, the jars closed and the original gaseous mixtures introduced and incubation continued. At the end of six weeks of incubation the jars were opened and all tubes were carefully examined microscopically and macroscopically. The data are presented in tables 4, 5, 6 and 7.

The tubes were taken from the jars and the surfaces of the mediums were carefully examined by transmitted and reflected light for colonies. When present the colonies were small, averaging about 1 mm. in diameter, and heaped up, with a distinct mucoid appear-

TABLE 5—*A comparison of the growths obtained on the mediums used for the isolation of B. leprae in various concentrations of O_2 and CO_2 in Novy jars incubated at 37°C. for 6 weeks*—Patient 1a*

Mediums	Percentage of CO_2 and O_2				Air Controls
	O_2 10.0; CO_2 10.0	O_2 20.96; CO_2 10.0	O_2 40.0; CO_2 10.0	O_2 0.0; CO_2 10.0	
Glycerol potato	?	?	+	0	0
Glycerol broth potato	0	?	+	0	0
Petroff's egg	+	+	+	0	0
Dorset's egg	0	0	+	0	0
Hormone glycerol agar	?	+	+	0	0
Plain glycerol agar	0	Cont.	Cont.	0	0
Rabbit blood agar	0	0	0	0	0
Rabbit serum agar	?	?	+	0	0
Glucose brain broth	0	0	0	0	0
Koori Konnyaku	0	0	0	0	0

* See footnote, table 4.

TABLE 6—*A comparison of the growths obtained on the mediums used for the isolation of B. leprae in various concentrations of O_2 and CO_2 in Novy jars incubated at 37°C. for 6 weeks*—Patient 2*

Mediums	Percentage of CO_2 and O_2				Air Controls
	O_2 10.0; CO_2 10.0	O_2 20.96; CO_2 10.0	O_2 40.0; CO_2 10.0	O_2 0.0; CO_2 10.0	
Glycerol potato	0	0	0	0	0
Glycerol broth potato	+	?	0	0	0
Petroff's egg	+	?	+	0	0
Dorset's egg	0	0	?	0	0
Hormone glycerol agar	0	?	+	0	0
Plain glycerol agar	Cont.	Cont.	0	0	0
Rabbit blood agar	0	0	0	0	0
Rabbit serum agar	0	0	0	0	0
Glucose brain broth	0	0	0	0	0
Koori Konnyaku	0	0	0	0	0

* See footnote, table 4.

ance and a loose filamentous border. Whether or not colonies were present, at least two smears were made with material taken from the surface of the medium and from the water of condensation in each tube. The preparations were air-dried, gently fixed and stained

with carbo-fuchsin, decolorized with 10 per cent H_2SO_4 and counterstained with Loeffler's methylene blue. Tubes which showed the presence of colonies and well formed, solid staining rods were designated as plus, there was a total of 46 tubes in this group. Frequently in the absence of colonies the smears contained well formed, solid staining rods suggestive of proliferation or at least of a favorable

TABLE 7—*A comparison of the growths obtained on the mediums used for the isolation of B. leprae in various concentrations of O_2 and CO_2 in Novy jars incubated at 37°C. for 6 weeks*—Patient 3*

Mediums	Percentage of O_2 and CO_2				Air Controls
	O_2, 10.0; CO_2, 10.0	O_2 20.96 CO_2, 10.0	O_2, 40.0; CO_2, 10.0	O_2, 0.0; CO_2, 10.0	
Glycerol potato	0	+	+	0	0
Glycerol broth potato	0	0	?	0	0
Petroff's egg	0	+	+	0	0
Dorset's egg	0	0	0	0	0
Hormone glycerol agar	+	+	?	0	0
Plain glycerol agar	0	0	Cont.	0	0
Rabbit blood agar	0	0	0	0	0
Rabbit serum agar	0	0	0	0	0
Glucose brain broth	0	0	0	0	0
Koori Konnyaku	0	0	0	0	0

* See footnote, table 4.

reaction to the new environment, such tubes were noted as containing a questionable growth. When the preparations contained only granular acid-fast bodies or highly granular rods, zero growth was recorded. No bacillus, coccus or actinomyces types were observed in any of these tubes or in the subsequent cultures excepting the aerobic spore-forming contamination noted in the ordinary glycerol medium.

A comparison of the data presented in table 4 should be made. It will be noted that no one medium or gaseous environment gave uniformly positive results. However, there seemed to be a distinct advantage in the use of the glycerol potato, egg containing and the hormone glycerol agar medium. The most favorable gas environment seemed to be 40 per cent O_2 and 10 per cent CO_2 although in the presence of 10 and 20 per cent O_2 plus 10 per cent CO_2 there were many positive results. It was rather to be expected from the experiments of Wherry that the low O_2 and 10 per cent CO_2 environment

would give the best results but such was not the case. The observed fact that 40 per cent O_2 and 10 per cent CO_2 was the most favorable environment is in accord with previous work on the tubercle bacillus wherein it was found that this increased tension of O_2 was the most favorable for the growth of this germ.

A striking fact is that no growth took place in any of the air controls or in the tubes incubated under anaerobic conditions plus CO_2. The air controls should be compared with the tubes incubated in air plus 10 per cent CO_2 which mixture seemed to be the second best for the isolations. Thus, one is to infer that the presence of free CO_2 is beneficial for the obtaining of primary isolations. From the absence of growth in the O_2 free jars, and it might be mentioned at this point that the granulation of the cells was most conspicuous in those tubes in the anaerobic jars, it may be inferred that the leprosy bacillus is a strict aerobe. It is possible, indeed later experiments have demonstrated, that the germ can utilize free oxygen at concentrations as low as 1 per cent if CO_2 is present and providing of course that sufficient O_2 is available at this low tension.

The growth from 16 of the tubes marked plus was taken up in physiological salt solution and this suspension, rich in acid-fast organisms, was used for the inoculation of the monkeys (see table 8).

Fresh potato, egg and hormone glycerol agar mediums were prepared and the growth from the remaining 30 positive tubes was transferred with a platinum wire to the freshly prepared mediums, the growth on each medium was transferred to a like tube of medium. After flaming the cotton plugs and cooling, the tubes were returned to the jars and the gaseous mixtures which had favored the initial growths were introduced. The jars were then placed in the incubator at 37.5° for four weeks. At the end of the incubation period the jars were opened and the tubes were examined macroscopically and microscopically for growth as before. Only 11 tubes in this second generation gave the typical colonies observed in the primary isolations and again the 40 per cent O_2 and 10 per cent CO_2 mixture seemed to be the most favorable atmosphere, the positive cultures were about equally distributed among the three varieties of mediums.

The growth was removed from the eleven positive tubes and placed on the surface of freshly prepared tubes of medium and returned to the jars with their former gaseous mixture and incubation again carried out for four weeks. It was noted on the examina-

tion of these tubes at the end of the incubation period that colonies were present in ten of the tubes but these so-called third generation colonies were no larger than the colonies obtained in the primary isolations, in other words the germs were not rapidly adapting themselves to a saprophytic existence.

Subcultures were made from the ten tubes to like medium and the freshly inoculated tubes were returned to the same gaseous environments, incubated as usual and examined at the end of four weeks. When the jars were opened only five of the tubes showed the typical colonies. These positive tubes were subcultured to about 20 tubes of each of the three favorable mediums and the freshly inoculated tubes were placed in the jars and 40 per cent O_2 and 10 per cent CO_2 were introduced, and incubation carried out as before.

The serial cultivation has been continued under the conditions outlined above and the germs are now in their ninth transfer on artificial medium. Generation five gave 8 positive cultures; genera-

TABLE 8—*Inoculation of monkeys with cultures of B. leprae*

Monkey number	Inoculated intradermally with culture suspension of B. Leprae	Result
	cc.	
M-1	0.5	Small nodule 21 days
M-2	0.5	Negative
M-3	0.5	Negative
M-4	0.5	Small nodule 7 days. Much larger 14 days. Excised
M-5	0.5	Negative
M-6	0.5	Small nodule 7 days
M-7	0.5	Negative
M-8	0.5	Negative
M-9	0.5	Small nodule 7 days. Much larger 14 days. Excised
M-10	0.5	Small nodule 7 days. Much larger 14 days. Excised
C-1	0.5	Small nodule 7 days
C-2	0.5	Small nodule 7 days. Much larger 14 days.
C-3	0.5	Small nodule 14 days
C-4	0.5	Negative
C-5	0.5	Small nodule 7 days. Much larger 14 days. Excised
C-6	0.5	Small nodule 7 days
C-7	0.5	Negative

tion six gave 6; generation seven gave 6, and generation eight gave 7 positive cultures. Starting with the inoculation of the material from the seventh generation, only hormone glycerol agar has been used. When transferring the organisms from the sixth generation several tubes of the hormone glycerol agar were enriched with 0.1 per cent asparagin; 0.05 per cent cystein; 0.05 per cent alanine and inoculated. An examination of the tubes at the end of the four weeks incubation period indicated no favoring action on the part of the amino acids. This is the status of the cultivation experiments to date.

A recapitulation of these experiments shows that there were 46 positive cultures in the first generation; 11 in the second; 10 in the third; 5 in the fourth; 8 in the fifth; 6 in the sixth; 6 in the seventh and 7 in the eighth. This data is suggestive of a gradual loss in the power of adaptation of this acid-fast organism to growth on artificial mediums. The cultural studies are being continued.

We believe these experiments confirm the recent work of Shiga (22) and also of Wherry (17) on the isolation of an acid-fast organism from human leprosy with perhaps much more satisfactory cultural data.

EXPERIMENTAL LESIONS IN MONKEYS WITH CULTURES OF B. LEPRAE

With definite proliferation of acid-fast bacilli in cultures prepared with the leprosy nodule emulsions it remained to determine if nodular lesions could be produced experimentally in monkeys with the artificial cultures of the organisms. Ten young *Macacus rhesus* and seven young *Cebus olivaceous* monkeys were inoculated intradermally over the eyebrow with 0.5 cc. of a pooled suspension of acid-fast bacilli coming from several cultures. Guinea pigs were also inoculated intraperitoneally with 1 cc. of this suspension to rule out *B. tuberculosis.* The results of the inoculations with cultures of the acid-fast bacilli from leprosy nodules grown on artificial mediums are found in table 8.

Five of the *Macacus rhesus* and five of the *Cebus olivaceous* monkeys which were inoculated intradermally with cultures of acid-fast bacilli developed nodules varying in size from that of a small pea to 1 cm. in diameter in from one to two weeks following inoculation. The nodular lesions were firm and hard, somewhat reddish in appearance and tended to regress rapidly after the third or

fourth week. There was no evidence of secondary infection or suppuration. From Cebus No. 1 smears were prepared from the nodule over which the skin was broken by injury in handling the animal and these smears showed numerous acid-fast bacilli with some mononuclear and polymorphonuclear cells. Sections of the nodules excised for histological study showed definite granulomatous changes.

One of these showed a marked cellular infiltration consisting of large mononuclears, polymorphonuclear cells and lymphocytes; marked oedema and an occasional multinucleated giant cell. The central cytoplasm of some of the giant cells was stippled but not definitely foamy in character. Acid-fast organisms could not be demonstrated in these sections. In other sections of nodules produced by inoculating cultures we have seen occasional acid-fast bacilli but they have been remarkable for their scarcity, although some of the sections removed on the thirty-first day have shown cells which were definitely becoming foamy in character, or else, as von Glahn has suggested, the cells were shrunken due to fixation and staining. These lesions, however, as the lesions produced experimentally in monkeys with *direct* inoculation of leprosy material from lepers, and which are described in the beginning of this report, are very early lesions. Our experience with such lesions, however, is limited and no studies are available in the literature on the histology of such early lesions. We feel therefore that both types of lesions should be classified as granulomas which are suggestive of early lesions in leprosy.

COMMENT

The reports of other investigators on the experimental production of nodular lesions of the granulomatous type in monkeys following inoculation with human leprosy material seems to be well founded. Our experiments would indicate that one may with a great deal of regularity infect certain monkeys with such material by intradermal inoculation and produce lesions within three weeks at the site of injection which are suggestive of lesions of early leprosy. Acid-fast bacilli are to be found in such lesions as other workers have observed. But in our experience they are few in number or entirely absent. The entire question of the cultivation of *B. leprae* should be studied further, ultilizing recent methods which have been advocated. Our experiments have led, we believe, to the unquestionable cultivation of *B. leprae* on artificial mediums. Experimental lesions have been reported by several investigators in such

animals as guinea pigs, rats, the Japanese dancing mouse, and other mice. None of these have been definite enough to permit their acceptance as evidence of experimental leprosy in these animals. Clegg was unable to infect monkeys with his cultures although guinea pigs were found to be susceptible. The lesions we have produced in two species of monkeys are very similar, if not identical, to those which other observers have been able to induce in these animals with leprosy material direct from human cases of the disease. But in addition we have also produced similar lesions in experimental monkeys with cultures of the organism. Apparently most of the acid-fast organisms injected, whether in human leprosy material emulsions or from cultures, are digested, absorbed, or otherwise disposed of by the tissues of the experimental host. It is frequently very difficult if not entirely impossible to demonstrate their presence in these experimental lesions. The observation is suggestive of both the low virulence of the organism and the high resistance of host. On the other hand in this series of monkeys inoculated with cultures Macacus monkey No. 10 developed a secondary lesion three months later at the site of inoculation from which a nodule was removed previously. Acid-fast organisms were demonstrable in smears from the secondary ulcerated lesion which indicates that in this particular animal the microbe possessed invasive properties and was capable of producing further lesions.

De Souza-Araujo, in his recent paper, has described experiments in which the inoculation of mice with boiled lepra emulsion containing acid-fast bacilli was followed later at autopsy by the finding of intact organisms, isolated in bundles or in globies. It will be recalled that Iwanow (23) in 1902 demonstrated the fact that guinea pigs inoculated with fresh and heated leprosy material developed similar lesions. In only those animals inoculated with fresh material, however, did the bacilli multiply. Fraser and Fletcher (24) have also reported nodular lesions in guinea pigs following inoculation with Kedrowsky's bacillus which had been previously killed. Such observations as the ones mentioned raise considerable doubt if experimental lesions such as have been reported in various animals are actually due to multiplying living organisms. In the experiments described in this paper we have no evidence that the acid-fast bacilli inoculated into monkeys remained alive in the animal's tissues. We do, however, know that actual multiplication took place in our culture mediums. Further we have pointed out

that in sections of the various lesions we have found a great scarcity of microbes, often finding none at all. The question arises, are we justified in assuming that, on the basis of positive cultivation of acid-fast bacilli from the fresh leprosy nodules, the organisms inoculated into animals were living at the time of introduction into the skin? Subsequent events consisted of the formation of granulomatous lesions within a relatively short time, and, in most cases, the disappearance of the microbes in the lesions. Were living organisms involved in the development of these lesions? Or did the destruction of living or dead organisms, or both, release some toxic substance from the bodies of the bacilli to which the tissues of the animal reacted by producing granulomas? Such speculations are at least suggestive of the possible mechanism by which human lesions may be produced in the long slow course of the disease. The chronicity of the human infection of leprosy may possibly be due, in part, to such a phenomenon. We offer then the following conclusions of experiments described in this paper with the limitations and reservations mentioned.

SUMMARY

The experiments described include (1) the experimental production of granulomatous lesions suggestive of early lesions of leprosy in two species of monkeys by intradermal inoculation of human leprosy material; (2) the cultivation of acid-fast (presumably *B. leprae*) bacilli from human leprosy nodules on several artificial mediums in various gaseous environments; and (3) the experimental production of granulomatous lesions, suggestive of early leprosy, in two species of monkeys by the intradermal inoculation of cultures of acid-fast bacilli from human leprosy material grown on artificial mediums. We believe the experiments indicate a step forward in the fulfillment of Koch's postulates for the causative agent in the disease of leprosy.

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DESCRIPTION OF PLATE

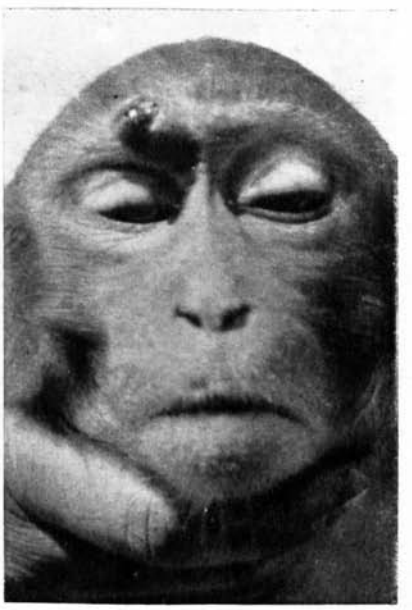
PLATE 1.

FIG. 1. Typical nodule on *Macacus rhesus* following inoculation with human leprosy material. Nodule developed in eighteen days.

FIG. 2. Same monkey as in Fig. 1 showing ulceration of nodule eleven days after appearance of nodule.

FIG. 3. Section of ulcerated nodule from monkey shown in Figs. 1 and 2 showing granulomatous lession.

1



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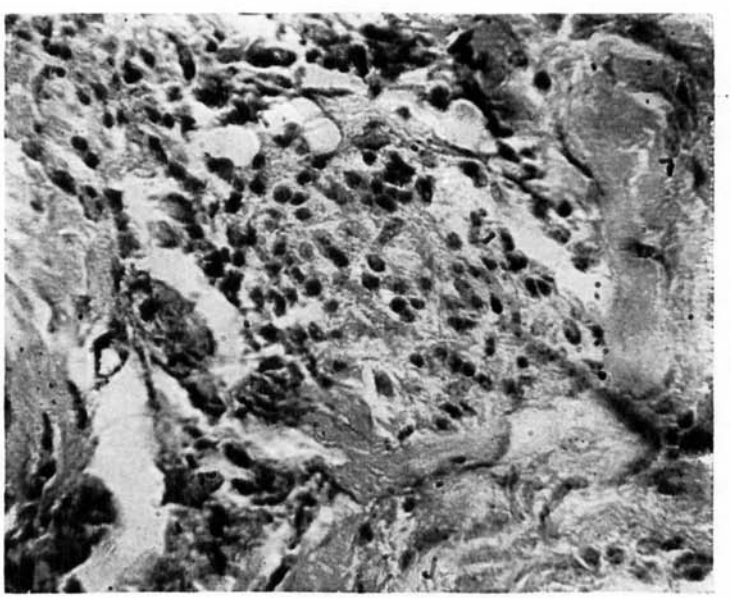

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PLATE 1.

DESCRIPTION OF PLATE

PLATE 2

FIG. 4. Typical nodule on *Cebus olivaceous* monkey following inoculation with human leprosy material. Nodule developed twenty days after inoculation.

FIG. 5. Ulcerated nodular lesion on *Cebus olivaceous* monkey shown in Fig. 4 which developed one week after appearance of nodule.

FIG. 6. Section of nodule from *Cebus* monkey shown in Figs. 4 and 5, showing granulomatous lesion.

4

5

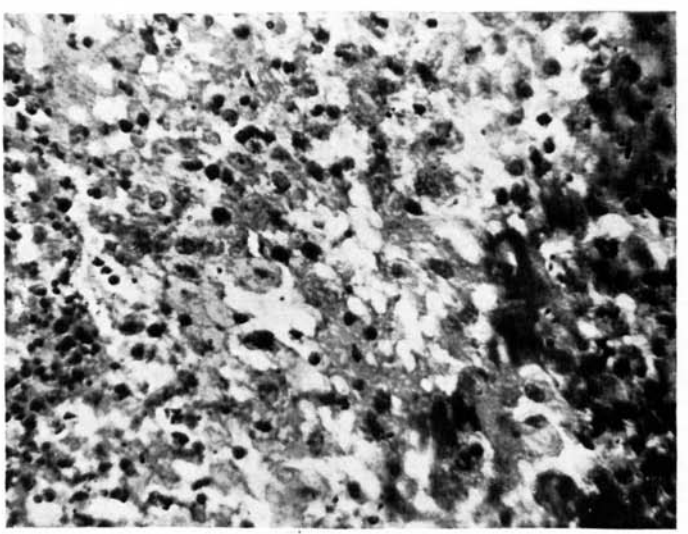

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PLATE 2.

DESCRIPTION OF PLATE

PLATE 3

FIG. 7. Typical nodule in skin of *Macacus rhesus* monkey produced with culture of acid-fast organisms from human leprosy material.

FIG. 8. Section of nodule from monkey shown in Fig. 7 showing granulomatous lesion and beginning foam cell formation.

FIG. 9. Ulcerated nodular lesion in skin of *Macacus rhesus* monkey shown in Figs. 7 and 8 produced with cultures of acid-fast bacilli from human leprosy material.

FIG. 10. Section of ulcerated lesion of monkey shown in Figs. 7 and 9, showing granulomatous lesion.

7

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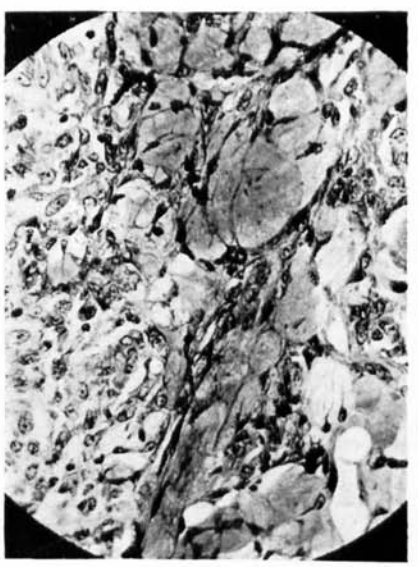

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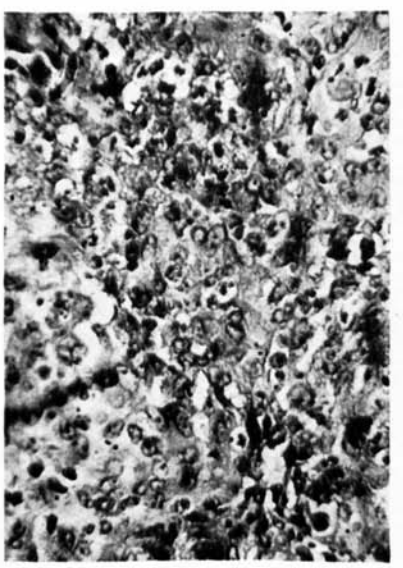

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PLATE 3.

DESCRIPTION OF PLATE

PLATE 4

FIG. 11. Nodule on *Cebus olivaceous* monkey produced with cultures of acid-fast bacilli from human leprosy material.

FIG. 12. Section of nodule produced with cultures in the skin of a *Macacus rhesus* monkey showing tubercle formation.

FIG. 13. Lepers from whom lepromata were excised for study.

11

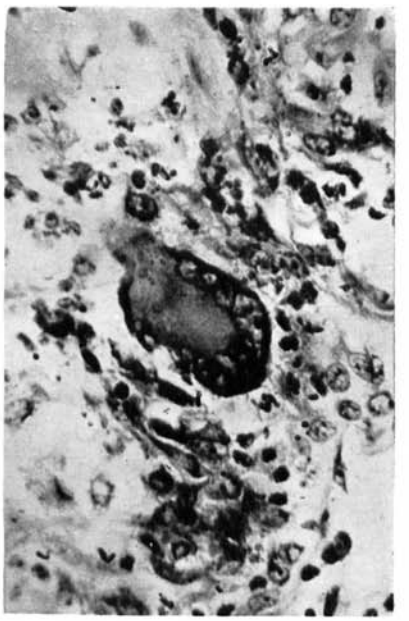

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PLATE 4.