

## ATTEMPTED CULTIVATION OF *MYCOBACTERIUM LEPRAE*

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Leprosy is a curious anomaly in the horde of diseases apparently caused by bacteria. It is the earliest known instance in which the supposed etiological agent was seen in the tissues of the infected host (10), or in which a bacterium was associated with a disease. In advanced lepromatous leprosy the number of these organisms in the tissue may be so great that a large portion of the cells of the lesions appears to be replaced by masses of them. In such cases they are not confined to the skin, but are also found in the liver and spleen in large numbers, as well as in the lymph glands, in the mucous membrane of the mouth and nose, in the nerves, etc. It is rare indeed for an infectious agent to be present in the specific lesion of a disease in anything like the numbers found in leprosy.

Faced with such an apparent wealth of material it would appear that this, of all the supposed bacterial diseases, would be the first to yield up its mystery to the processes of cultivation and experimentation. As is natural, attempts to grow these acid-fast bacilli have been made since the beginning of bacteriology. Gaucher and Hillairet apparently reported the first attempt in this direction in 1880 (9, 11). Their culture was a filamentous organism which must have been a contaminant; at all events, it was not regarded seriously by later workers. The attempts to isolate and grow this parasite away from its host have continued to the present time, by many workers in all parts of the world. This work has probably been reviewed more comprehensively by McKinley (23) than by anyone else.

In one important group of experiments living tissue was made use of. Timofejewsky (34) claimed to have obtained continued multiplication of *Mycobacterium leprae* in the macrophages of human tissue culture. Salle (27), and Salle and Moser (30) also isolated, by means of chick tissue culture, an organism which they believed to be the etiological agent. The same organism was also isolated by them in fresh minced chick medium, and in veal glycerine agar.

It consisted of a diphtheroid which was acid-fast in young cultures containing living or fresh tissue, and nonacid-fast in old cultures or those grown on autoclaved media. The acid-fast characteristics appeared again on transfer to minced chick medium (28), or on other special media (29). Walker and Sweeney (35) isolated an acid sensitive, facultatively acid-fast organism in 13 percent of their cultures from rat material, but obtained no multiplication of acid-fast forms. Duval (5) observed the changes which took place in tissue cultures of leprosy material over a period of six months. Lowe (20, 21), on the other hand, failed to obtain growth by the tissue culture methods of Timofejewsky and Salle.

Another group of workers has made serious attempts to cultivate this organism from the blood. Loewenstein (17, 18, 19) applied to leprosy the method he used for blood culture in tuberculosis. By means of a special fish medium, he claims to have isolated acid-fast organisms regularly from the blood of lepers. Jordan (12) also claims to have isolated an acid-fast bacillus from blood. Lleras-Acosta (16) used a method quite similar to that of Loewenstein and isolated an organism which resembled the latter's in some respects; the medium used was a modification of Petragani's potato-milk-egg medium. The work of Loewenstein has been repeated by others with little or no success. Lépine, Markianos and Papayoannou (14) failed with this method, using both blood and leprosy as inocula. Souza-Araujo (32) and Dubois, Gavrillov and Fester (4) failed to obtain cultures from the blood of lepers by this method. Solana and Gutierrez-Solana (31), Eddy (7), Lie (15) and Lowe (21) have all failed to obtain from blood or lepromata, by any method, cultures which they believe to be the Hansen bacillus. Some of the reported positive results may be explained by the work of Aoki (1), who obtained from water an orange-yellow mycobacterium which was very resistant to sulfuric acid.

McKinley and his associates (23, 24) claimed to have isolated a delicately growing acid-fast bacillus by means of a modification of Wherry's gaseous tension method. They found 10 percent carbon dioxide and 4 percent oxygen to give the best results. This method has been repeated by others, including Lowe (20, 21), Salle and Moser (30), Eddy (7), and Duval and Holt (6). None of them were able to grow an organism resembling that obtained by McKinley.

In the opinion of Lowe, the many negative attempts to cultivate the Hansen bacillus which have been made and are being

made should be reported. Although it is usually assumed that in most fields a positive result is worth more than many negative ones, in the field of bacteriology negative results often tend to disclose errors in technique or in observation which have a tendency to become accepted as facts. Such reports may also prevent waste of time in repeating work that has already been thoroughly checked.

#### METHODS AND PROCEDURES

In attempting to attack this difficult problem, it is essential to consider what is acceptable as fact, or what is actually known about the etiological agent of this disease. In the first place, when *M. leprae* is present in a living organism (i.e., human being or rat) it usually multiplies extensively, sometimes to an unbelievable degree. Secondly, it can safely be concluded that this organism will not multiply to an appreciable extent on ordinary laboratory media under ordinary conditions of aerobiosis or anerobiosis and at ordinary temperatures of incubation. Thirdly, it is extremely doubtful that this organism has yet been cultivated by use of special media or special methods of incubation. The chief criticisms that are applicable to the reported positive results are (a) lack of uniformity, the percentage of positive cultures in any series being very low; and (b) lack of confirmation by other experimenters attempting to repeat the work.

The specimens used in these experiments were all taken from living patients under strictly aseptic conditions. Blood was obtained from the cubital vein after painting the skin with tincture of iodine. It was delivered immediately into a sterile test tube containing sodium citrate as an anticoagulant. Culture media were inoculated with whole blood, six to eight drops being placed in each tube by means of Pasteur pipettes. Smears were made from the whole blood, and also from the leucocyte layer obtained by allowing a small amount of blood to settle for several hours in a slender tube in the ice box, according to Moutoussis' method (25).

Most of the tissue specimens were nodules, selected lesions covered by intact skin and not contiguous to ulcerated areas or areas that showed evidence of any infection other than leprosy. The nodules were removed under strict surgical asepsis. If the lesion was subcutaneous, it was removed through an incision in the skin. If it was cutaneous, the epidermis covering it was removed by electric cautery and the nodule excised through the cauterized area, though in one instance such a leproma was removed with its skin after first disinfecting with tincture of iodine and alcohol. In all cases the excised tissue was placed in a sterile petri dish and taken to the laboratory, chopped up with sterile scissors into a mortar provided with a paper apron (8), and then ground with sterile washed quartz sand and sterile saline solution. The resulting emulsion was used immediately for the inoculation of media. The inoculum was quite large, four to six drops from a Pasteur pipette being used for each tube.

Eighteen specimens of blood were examined and cultured. Fourteen of them were obtained during acute leprosy reaction. The other four were obtained from a group of patients that had been given a series of injections of tuberculin. One-third of these blood specimens were positive for acid-fast bacilli when concentrated, and one specimen contained so many that they were easily found on direct smear of unconcentrated blood. Thirty-nine specimens of tissue were used in these experiments, as well as four specimens of pus from lesions not typical of those caused by pyogenic organisms. One blood sample was obtained from a pure neural-type case, but all of the other specimens were taken from cases of nodular or mixed type.

Throughout this work emphasis has been placed on asepsis. In no case was a specimen treated chemically or by heat in a manner that might have destroyed living organisms. In only one instance was a bacteriostatic or inhibitory reagent other than glycerine used in a culture medium for primary inoculations; this was in the case of a Corper's potato medium which contained crystal violet. Chances of extraneous microorganisms gaining entrance were minimized in several ways. All solutions and reagents used in the preparation of tissues were autoclaved at fifteen pounds (121°C) for twenty minutes. All glassware and equipment used in preparing media or specimens was autoclaved at twenty pounds (126°C) for twenty minutes and dried in the hot-air oven. All media to which the regular sterilization procedure could not be applied, such as coagulated egg media, were prepared under conditions as nearly aseptic as possible, and autoclaved at the temperature stated, pressure being used with a low temperature (i.e., air-steam mixtures). Controls for sterility of all media were made by incubation tests. Inoculations were carried out in a glassed cabinet previously washed down with a disinfectant.

A large number of different media were used. Some of these have not been used extensively in leprosy work, and others were modifications of media that have been more or less accepted as favorable for growth of the acid-fast group of bacilli.

One group consisted of media prepared according to the formula of Lawson (13) from *dried legumes*.<sup>1</sup> The formula was followed precisely, except that

<sup>1</sup>Soak 200 gm. of dried legumes in 700 cc. of water for 24 hours. Change water and autoclave at 15 pounds for 25 minutes. Drain off liquid and mash legumes through a fine sieve. Mix 30 cc. of legume liquid with 180 cc. of distilled water. Mix liquid with 105 gm. of purée of legume. Put in 500 cc. flask and add 3 gm. of granulated agar. Autoclave at 15 lbs., 20 minutes. Add 7 cc. of glycerine and appropriate dye. Cool to 50-60°C and pour aseptically. The tubes must be chilled thoroughly and, when filled, slanted in ice and water to insure rapid solidification. Use within 48 hours.

no dyes or other bacteriostatic agents were added. The following legumes were used: navy beans, red kidney beans, cow peas, black-eyed peas, and lima beans.

Milk was used for the basis of a second group of media. Fresh milk, obtained by hand-milking into a sterile flask, was delivered immediately to the laboratory, neutralized to pH 6.8 to 7.0, tubed and sterilized at 15 pounds pressure for 15 minutes. Dextrose (0.5 percent) was added to some batches before tubing, and maltose (0.5 percent) was added to others. Milk agar was prepared by adding 1.5 percent of agar to the milk, dissolving it by careful boiling, tubing and autoclaving as above. A mash medium was prepared by adding purée of legumes (black-eyed peas and lima beans), *M. phlei* suspension, sugars (dextrose and maltose), and glycerine to fresh whole milk. It was tubed and autoclaved at 15 pounds pressure for 15 minutes, as described.

*Milk-egg mixtures* were used in two basic media. One consisted of fresh boiled milk mixed aseptically with an equal part of whole egg and five percent of glycerine. It was sterilized by autoclaving at 85° to 90°C at ten to fifteen pounds pressure for 45 minutes.<sup>2</sup> The other consisted of a mixture of equal parts of fresh boiled milk and the yolk of eggs. It was prepared and tubed aseptically and sterilized as described.

Media of which the basic constituent was *egg* consisted, in addition to the above egg-milk mixture, of Dorset's egg medium prepared as usual except that the amount of liquid was doubled, and the egg yolk medium of McCoy and Chapin (22).

Modifications of some of the above media were made by substituting 5 percent of glycerine for an equal amount of fluid. The milk-whole egg-glycerine medium has already been mentioned. Dorset's egg medium with glycerine, milk-egg yolk with glycerine, and the egg yolk medium of McCoy and Chapin with glycerine were also used. The legume agar contains about 2.3 percent of glycerine.

Enrichment of culture media by the addition of killed acid-fast bacilli has been made use of in the isolation of *M. paratuberculosis*, and has been tried in leprosy work. This method was attempted, using the following preparation. *M. phlei* was grown for one month in flat flasks containing a shallow layer of glycerine beef-infusion broth. At the end of this time the liquid had evaporated to about one-half of the original volume and a luxuriant growth of the culture was present. The flasks were shaken and the contents poured into an Erlenmeyer flask. The pooled culture suspension was shaken with glass beads until well emulsified and then autoclaved at fifteen pounds pressure for 15 minutes.

Legume agar was modified by substituting\* 10 cc. of the *M. phlei* suspension for 10 cc. of distilled water in the original formula. This resulted in a 3 percent concentration in the finished medium. The following *legume-M. phlei* media were prepared: navy bean, kidney bean, cow peas, black-eyed pea, and lima beans. Corper's potato slants were prepared and mois-

<sup>2</sup>The autoclave was run up to 100°C with the valves open. Steam was then cut off and, as soon as pressure was zero, the door was opened and material to be sterilized put in. The door and all valves were closed and the steam valve opened. The temperature had to be regulated by manipulation of the steam valve during the entire sterilization period.

tened with 1 to 2 cc. of the suspension in place of broth. This preparation was substituted for fluid to make a final concentration of 5 percent in the following media: Dorsett's egg medium, egg yolk medium of McCoy and Chapin, milk-egg yolk medium, and milk-whole egg medium. Both glycerine and the *M. phlei* preparation were used in 5 percent concentrations of each in the following media: egg yolk medium of McCoy and Chapin, milk-egg yolk medium, and Dorsett's egg medium. The legume-milk-sugar mash medium previously referred to contained approximately 10 percent of *M. phlei* preparation and 2.5 percent glycerine.

A miscellaneous group of media which had little in common were also used. These comprised the following: nutrient agar, glycerine agar, Corper's potato medium, minced chick embryo medium of Salle, dextrose brain broth of Rosenow (26), and dextrose brain semisolid agar. This last was prepared by adding 0.5 percent of agar to the broth used in making the dextrose brain broth.

The temperature of incubation was 37°C for all cultures for the first three months. The majority were incubated at this temperature for the entire experimental period, but about 200 tubes were kept at room temperature from the fourth month to the termination of the experiment. The tubes were closed with sealing wax put on over the cotton stopper to prevent drying. No attempt was made to create anaerobic conditions. However, dextrose brain agar is a satisfactory medium for cultivating anaerobes as well as aerobes, and the depth of dextrose brain broth will support the growth of some anaerobic and microaerophilic organisms. It is probable that conditions approaching anaerobiosis were present in the butts of the legume agar slants, because cultures grew there that exhibited microaerophilic characteristics when studied on other media.

The total number of individual cultures inoculated was 1,718. These were observed for different lengths of time up to thirty-six months. Tubes were discarded when they became dried out or when examination showed that the acid-fast organisms had disappeared. Most of the culture tubes that were opened from time to time for routine smears were discarded. Over one-half of the cultures (942 tubes) were observed for twenty-two months or more; of these, 573 were observed for thirty-six to thirty-nine months. Each specimen was used for planting from 5 to 250 tubes of media. The total number of tubes of any one medium used varied from 20 for milk agar to 327 for black-eyed pea agar. On an average, 50 tubes of each medium were inoculated with emulsion from leprous lesions or with blood.

#### RESULTS

Smears made from cultures from time to time failed to show any definite evidence of growth of the acid-fast organisms. In some cases the number of bacilli appeared to have increased, but this was never very marked and could be explained by concentra-



tion due to evaporation, or by a peculiar behavior of the inoculum. The minute tissue fragments would form aggregates very much like those formed in the agglutination of bacteria. This process apparently went on for twenty-four to forty-eight hours after the emulsion was prepared. In a tube containing inoculum alone, it resulted in a clear supernatant fluid with a tough, rubbery clot at the bottom which contained most of the organisms. In a tube of medium the texture of the clot was modified by the composition of the water of synuresis. Usually the clot formed at the base of the slant was softer and more friable than that formed in the inoculum alone. Smears made from this material at the base of the slant contained larger numbers of organisms than smears made from the surface of the slant or from the supernatant liquid.

Changes in the morphology of the acid-fast organisms were observed quite frequently. In most cases this was not marked and could be accounted for either by assuming that the bacilli were undergoing disintegration or that they had been liberated by the breaking up of a certain number of the globi and large clumps present in the original inoculum. These assumptions were based on strongly suggestive appearances on the slides. In one instance an entirely new form appeared in the tubes of legume agar. This was a brilliant, solid-staining organism, curved to form a portion of the arc of a circle, and occurring in long, tangled, rope-like arrangements. Subcultures from these tubes on the same and different media failed to show any increase in numbers. There was no macroscopic indication of growth.

The acid-fast organisms from leprous tissue appeared to be very durable on culture media. In most tubes, up to thirty-six months after inoculation, there appeared to be approximately the same number of organisms as were present after one month of incubation. A few tubes showed a marked decrease in numbers for the first time when they were about twenty months old. In some of these tubes acid-fast forms appeared that were apparently degenerated *M. leprae*. The most common one was a heavy granule with a ghostly rod extending from it. Another form observed was a pale globule, which occurred with typical rods. It was noted that in some tubes which showed growths of staphylococci, the acid-fast bacilli tended to disappear. This happened quite rapidly. It was also difficult to find the original inoculum in tubes that were overgrown with mold. Such tubes were discarded.

Media containing fat (i.e., egg media) had a tendency to form colony-like spots. Usually the clear yellow fat drops could be iden-

tified, but occasionally suspended matter of some kind gave them an opaque, creamy appearance. Smears of such structures showed about the same number of acid-fast organisms as were found on other portions of the slant. Subcultures were always negative.

The only cultures of acid-fast bacilli obtained in the entire series of experiments described here were two strains of *M. tuberculosis*. One was isolated from the pus from an abscess on the sternum, and the other grew from the cultures made from pus obtained from an abscess of the neck. The identification of both of these cultures was confirmed by guinea-pig inoculation. Incidentally, both of them grew out on Lawson's agar in twenty days.

In dealing with such a large number of cultures, it was inevitable that a certain amount of contamination incidental to inoculation should occur. The contaminations noted were of two types, fungous and bacterial, the former more common than the latter. Tubes in which molds were observed were discarded as soon as they became overgrown. The bacterial contaminations were observed in four tubes, occurring as single colonies or as pure cultures in isolated tubes of media. The organisms observed were: chromogenic, nonacid-fast, gram-negative, nonsporulating bacilli; nonacid-fast, gram-positive, sporulating bacilli; and sarcina.

One would expect that another source of contaminating bacteria in such experiments as these would be the skin itself. However, only one set of cultures showed growth that could be attributed to this source with a fair degree of certainty. The specimen used in this instance was a cluster of cutaneous nodules removed from the back of the neck after cauterizing the skin. The probable source of the organisms was one or more folds of skin which the disinfectant and cautery failed to reach. A mixed culture consisting chiefly of staphylococci and diphtheroids grew out on every culture tube inoculated with this emulsion. It is interesting to note that the number of acid-fast bacilli decreased rapidly in these tubes. Another specimen gave a growth of staphylococci in two out of eight tubes inoculated. They first appeared about five months after inoculation, and it is possible that they came from some other source than the tissue. The specimen was a single nodule removed after cauterizing the skin. Again the acid-fast organisms were found to be absent in the contaminated tubes.

In two instances microorganisms were observed in one tube of a set inoculated with whole blood. In both cases the organism was a minute, gram-negative, nonsporulating, microaerophilic cocco-



bacillus whose characteristics corresponded to those of the genus *Bacteroides*. This growth appeared in the cultures about five months after inoculation. It has been shown by Thompson and Henthorne (33) that these organisms are capable of invading tissue under some conditions. Burdon (3) found them on mucous surfaces, especially in the mouth. He showed that *Bacteroides melaninogenicus* increased markedly in numbers in disease, and under filthy conditions of the tissue; also that it could invade the blood stream from diseased tissue (uterus) along with the streptococcus. Considering these facts, and the delicacy of the organism isolated, it is probable that the source was the blood rather than the medium or the atmosphere.

Molds which apparently occurred in the tissue were found in two specimens. The first of these was a cutaneous nodule removed intact with its overlying skin after iodine-alcohol disinfection. The fungus grew out after about two weeks on every tube inoculated. The growth was of the *Epidermophyton* type. This is the only instance in which outer layer of skin was knowingly used. The second specimen showing fungus growth was the tip of an enlarged ear lobe removed after cauterizing the skin. The mold appeared on all of the cultures within ten days after inoculation and grew first as a snowy duvet which developed a covering of golden brown spore heads in about a month. These were of the *Aspergillus* type, but the chains of spores were long and glued together, so that the fruiting bodies appeared grossly as tiny golden sticks.

A slow-growing, microaerophilic diphtheroid grew out in cultures from five separate tissue specimens. The odd thing about these cultures is that they were all very much alike. The first specimen to show such a growth was an unusually large subcutaneous leproma that, when cut, was found to be broken down in the center. Smears, however, revealed large numbers of acid-fast bacilli and no blue-staining organisms. The diphtheroid grew out in six of nine cultures. The second specimen was a piece of tissue consisting of two cutaneous nodules which were very close together, with a depressed area in the center which may not have been thoroughly cauterized. Two out of five tubes showed growth. The third specimen was pus obtained from an acute abscess on the neck, which contained about 10 cc. of pus. The skin was disinfected with iodine before the lesion was lanced, and a small amount of pus was collected on a sterile swab as it exuded from the lance wound. This material, rich in acid-fast bacilli, was emulsified in about 1 cc. of sterile saline and used immediately for culture inoculation. The organism grew

out in four out of five tubes of media. The fourth specimen consisted of two small cutaneous nodules removed after cauterizing the skin; growth occurred in two out of eight tubes. The fifth specimen was a large subcutaneous nodule; the diphtheroid grew out in two out of eight tubes of media.

The characteristics of the organisms which were cultured from these five specimens would indicate that they were closely related, if not identical. Primary growth on legume agar appeared first as a white, irregular film at the base of the slant between the medium and the glass. In some cases the liquid at the base of the slant became granular. In dextrose brain broth there was a faint cloudiness, and in old cultures a slight ring at the top of the medium. Dextrose brain semi-solid agar showed a cluster of fleshy, pinkish colonies about one-half inch below the surface of the medium; these had the appearance of fragments of brain tissue. In old subcultures growth would finally come to the surface, and after several subcultures on this medium it became much more aerophilic in its habits. Growth was very slow in primary cultures. It was first observed about two months after inoculation in four instances, and in the fifth one (the pus specimen) after three weeks. Subcultures of all strains on dextrose brain semi-solid agar, which appeared to be the most favorable medium, grew out in ten to fourteen days. Microscopically these organisms were all nonacid-fast, gram-positive, small slender pleomorphic rods. They stained irregularly with methylene blue. They were similar in some respects to *Corynebacterium acnes* (Gilchrist) as listed in Bergey's Manual of Determinative Bacteriology (2).

#### DISCUSSION

The significance of any of the microorganisms which grew out in these cultures as an etiological agent in leprosy is probably nil. The two cultures of *M. tuberculosis* were the only recognized pathogenic ones isolated. The organisms which occurred in a single tube out of a number inoculated can only be regarded as chance isolations. Whether they came from the air or the tissue is immaterial, especially since none of them belonged to a class of bacteria that need be seriously considered as a possible etiological agent in leprosy. It should be noted that chance contaminations of cultures which would cause no trouble in ordinary bacteriological work occur in this type of experiment because of the extremely long incubation periods used. This gives slow-growing organisms time to make their appearance.

Of all the eleven tissue and pus specimens which yielded growths of similar character on two or more tubes, that from one specimen can be dismissed as gross skin contamination; growth from another was a staphylococcus; two gave pure cultures of fungi of such types that they need not be considered as causal organisms in this disease; and two others yielded pure cultures of *M. tuberculosis*. The diphtheroid organisms need a little more consideration because of the frequency with which such cultures have been isolated from leprous tissue, and because they constitute five of the eleven cultures obtained. An etiological relationship has sometimes been claimed for members of this group. In this instance, however, it is doubtful that they are important. One reason for this opinion is that such organisms have been found so frequently by competent workers in normal tissues, and as nonspecific secondary invaders in diseased tissues, that their occurrence is generally accepted. They are of special importance on the skin and mucous membranes. The diphtheroids isolated in these experiments correspond to a type frequently observed in the deeper skin structures, and sometimes in lymph glands, especially in their microaerophilic mode of growth.

#### SUMMARY

Under the conditions of these experiments, media prepared with legumes, milk, egg or egg yolk, *M. phlei*, or various combinations of these ingredients, failed to promote growth of the acid-fast bacilli from lepromas or the bloods of leprous patients. Neither was growth of these organisms obtained with minced chick embryo medium, Corper's potato medium, dextrose brain broth, or dextrose brain semisolid agar.

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