THE CULTIVATION OF THE LEPROSY BACILLUS FROM THE HUMAN TISSUES WITH SPECIAL REFERENCE TO THE AMINO-ACIDS AS CULTURE MEDIA.*

By CHARLES W. DUVAL, M.D.

(From the Laboratories of Pathology, Tulane University, New Orleans, Louisiana.)

In a previous communication,[1] I pointed out that although the leprosy bacillus will readily multiply in pure culture upon a variety of media, after it has once acquired more or less saprophytic properties the initial multiplication outside of the animal body is effected, on the contrary, with extraordinary difficulty and not at all unless special media and methods are employed. This paper aims to give briefly the results of my experience with the amino-acids in the cultivation of the lepra bacillus and to discuss the most advantageous methods of its isolation. There are several ways in which the organism may be cultivated from the tissues, but two in particular are worthy of special consideration and will be described in detail.

When special food stuffs are seeded simultaneously with some bacterial species which are known to aid the artificial growth of *Bacillus leprœ*, the latter increase in number very slowly and do not show macroscopic growth for several weeks, while generations removed from this parent stem multiply rapidly and reach under the same conditions a maximum growth in two to three days.

This wide difference in the cultural behavior of lepra bacilli under conditions of artificial environment is not unusual since other pathogenic microörganisms behave in a similar manner. In this respect *Bacillus leprœ* has its prototype in certain strains of the tubercle family which at first grow feebly, and in some instances not at all, on the most favorable artificial medium. The fact that the initial growth of the leprosy bacillus is so difficult to obtain

* Received for publication, January 13, 1911.

[1] *Jour. Exper. Med.*, 1910, xii, 649.

even in the presence of special foodstuffs, although once cultivated it grows profusely on ordinary media, is not surprising when we consider that *Bacillus tuberculosis* is incapable of cultivation directly from the tissues on glycerine agar, upon which medium it grows well in subsequent generations.

Rapid growth of *Bacillus leprœ* indicates, of course, frequent division of the bacterial cell and is a property quickly acquired by cultures that are no longer influenced by the conditions of the host. In the animal tissues, division of the leprosy organisms takes place slowly, and probably more slowly than that of the tubercle bacillus under similar conditions. In this connection it is noteworthy that the bacilli in the first generations on artificial media, like those in the tissues, multiply very slowly and are long, slender, and distinctly "beaded," while those several generations removed from the parent stem grow with great rapidity and are short, almost coccoid, and devoid of "beads." Since "beading" of the bacilli always occurs in the animal body and persists for a short time only in artificial cultures, it is not only possible but highly probable that the leprosy bacillus after all is not a true parasite in the strict sense of the term. The difficulty with which animals are infected, including the human subject, and the commensal relationship enjoyed by the bacilli in the tissues of man strengthens such an hypothesis. The transformation in morphology of the organism has been fully described in a recent paper.[2]

Clegg[3] was the first to show that the acid-fast bacilli in leprous tissue will multiply upon an artificial medium and continue to grow in subculture. His results were obtained with ameba and their symbiotic bacteria from which pure cultures of *Bacillus leprœ* were subsequently derived by heating at 60° C. for thirty minutes, which temperature destroyed the ameba and symbiotic bacteria without injuring the acid-fast organisms.

Soon after the publication of Clegg's paper, the author confirmed his results and, moreover, succeeded in the isolation and cultivation of *Bacillus leprœ* directly from the human tissue without the aid of ameba or bacteria upon a tryptophane medium. At this time

[2] *New Orleans Med. and Surg. Jour.*, 1911, lxiii, 549.
[3] *Philippine Jour. Sc.*, 1909, iv, 403.

it was noted that the specific organism of leprosy would multiply in and around the transplanted pieces of leprous tissue upon an ordinary blood agar medium which had been seeded with influenza bacilli or with meningococci. No attempt was then made to explain the occurrence of the growth of leprosy bacilli in association with other bacteria aside from the general notion that the transferred tissue was responsible. While the tissues do play a part, recent work tends to show that the associated bacteria play by far the more important part.

It can be definitely stated that the initial growth of *Bacillus leprœ* upon a medium of ameba and bacterial symbiotics is the result of the reaction of the associated bacteria; namely, *Bacillus typhosus, dysenteriœ, coli, cholera,* etc. This is contrary to the view first formed by me in a previous paper[4] in which I stated that the ameba in some way supplied the necessary nutriment for the lepra bacilli; recent tests, however, do not support this contention.

Experiments show that amebæ are in no way concerned in the multiplication of *Bacillus leprœ* and should not be employed in the culture medium. Growth of leprosy bacilli is not aided in the least by either the encysted or ameboid forms; but on the contrary the vegetative type of the protozoan may prove a serious hindrance to the initial growth of the bacilli, since they thrive better in symbiosis with pure cultures of leprosy than they do with any of the typhoid-colon group. Encystation does not occur as early with cultures of *Bacillus leprœ* and this may be attributed to the fact that leprosy bacilli live for months and are constantly multiplying, whereas the typhoid colon group of bacilli live for weeks only, and ameba appear not to feed upon dead cultures.

The ordinary laboratory media may be successfully employed to recover *Bacillus leprœ* if bits of tissue are carried over to the culture medium and bacilli such as the colon, typhoid, proteus, subtilis, etc., are added at the same time. If the tissue is already contaminated by bacteria there is no necessity for this addition, as the contaminators answer the purpose equally well.

The primary multiplication of *Bacillus leprœ* in excised bits of tis-

[4] *Jour. Exper. Med., loc cit.*

sues in the presence of the common bacterial species is caused by the chemical action of the extraneous bacteria on the cells of the tissue whereby the nucleo-proteids are split up and changed. These final products of tryptic digestion, of which tryptophane is one, are capable of being assimilated by the leprosy bacilli through which their growth is promoted. *Bacillus leprœ*, it would seem, is unable to use the albumen molecule as such, but can readily assimilate the split products. This explains why growth of the bacilli occurs when extraneous bacteria are present, and the failure to multiply where they are absent, unless some of the cleavage products are included as part of the artificial medium. In support of this explanation repeated tests have shown that growth will not occur where the lepra bacilli are first washed free from tissue cells before seeding with the other bacteria. On the other hand, the acid-fast bacilli in excised bits of leprous tissue that are contaminated with putrefactive bacteria, steadily increase in number when kept even at room temperature in distilled water or salt solution; while in uncontaminated leprous tissue under the same conditions the lepra bacilli will not multiply though they remain viable for months, as shown by subsequent cultural or animal tests.

It is well known that putrefactive bacteria and many of the intestinal species will cause disintegration of the albumen molecule with the formation of albumoses, peptone, and finally amino-acids. In other words, certain bacterial species behave towards the nucleoproteids in the same way as trypsin. Apparently *Bacillus leprœ* itself has no power to split or hydrolize the albumen moiety.

Obviously then two methods may be followed in the initial cultivation of *Bacillus leprœ* from the leprous tissue, (1) the use of a special medium containing amino-acids (direct method), and (2) the addition of some bacterium to the culture medium which is capable of hydrolizing the transferred bits of infected tissues (indirect or bacterial method).

The direct method has the advantage of simplicity and requires no subsequent plating or heating to obtain a pure culture and is applicable in all cases where pieces of tissues can not be obtained. On the other hand, though the indirect method requires no special

bacteriological technique to provide against contamination, it does necessitate subsequent replating or heating of the growth in order to separate *Bacillus leprœ* from the associated bacteria. Besides, it is also necessary to transfer the pure colonies to an amino-acid medium to insure continued growth until the culture is accustomed to the new environment. Although both methods have their advantages and disadvantages, the former is to be preferred for practical purposes.

METHOD OF CULTIVATION.

In a previous communication,[5] the use of tryptophane and other amino-acids was recommended for the direct isolation of *Bacillus leprœ* from the tissues, but as these substances are difficult to obtain and prepare I have recently used a medium composed of albumen and trypsin which gives equally good results. In this medium the necessary amount of digestion of the nucleo-proteid is secured by the trypsin. Its preparation and method of use follow: egg albumen or human blood serum is poured into sterile Petri dishes and inspissated for three hours at 70° C. The excised leprous nodule is then cut into thin slices, two to four millimeters in breadth and five-tenths to one millimeter in thickness, which are distributed over the surface of the coagulated albumen. By means of a pipette the medium thus seeded with bits of tissue is bathed in a 1 per cent. sterile solution of trypsin, care being taken not to submerge the pieces of leprous tissue. Sufficient fluid is added to moisten thoroughly the surface of the medium.

The Petri plates are now placed in a moist chamber at 37° C. and allowed to incubate for a week to ten days. They are removed from the plates from time to time, as evaporation necessitates, for the addition of more trypsin. It will be noted that after a week or ten days the tissue bits are partially sunken below the surface of the medium and are softened to a thick, creamy consistence, fragments of which are readily removed with the platinum needle. On microscopic examination of this material it is noted that the leprosy bacilli have increased to enormous numbers and scarcely a trace of the tissues remain. Separate lepra bacilli colonies are also discernible on and around the softened tissue masses, and those

[5] *Jour Exper Med, loc. cit.*

furthest away from the masses attain a size of two millimeters in diameter. The colonies at first are greyish white, but after several days they assume a distinct orange yellow tint.

From the plates subcultures may be obtained by transferring portions of the growth to a second series of plates or to slanted culture tubes that contain the special albumen trypsin medium. After the third or fourth generation, the bacilli may be grown without difficulty upon glycerinated serum-agar prepared in the following manner: twenty grams of agar, three grams of sodium chloride, thirty cubic centimeters of glycerin, and 500 cubic centimeters of distilled water are thoroughly mixed, clarified, and sterilized in the usual way. To tubes containing ten cubic centimeters of this material is added in proper proportion a solution of unheated turtle muscle infusion. Five hundred grams of turtle muscle are cut into fine pieces and placed in a flask with 500 cubic centimeters of distilled water. This is kept in the ice chest for forty-eight hours and then filtered through gauze to remove the tissue. The filtrate is then passed through a Berkefeld filter for purposes of sterilization. By means of a sterile pipette, five cubic centimeters of the muscle filtrate is added to the agar mixture which has been melted and cooled at 42° C. The tubes now are thoroughly agitated and allowed to solidify in the slanted position.

This medium is perfectly clear, or light amber in color, and admirably suited for the cultivation of *Bacillus lepræ* once the initial culture has been started. Growth is luxuriant and reaches its maximum in forty-eight to sixty-four hours. On the surface of this medium the growth is moist and orange yellow in color, while in the water of condensation, though growth apparently has not occurred, the detached bacilli collect in the dependent parts in the form of feathery masses without clouding the fluid.

Ordinary nutrient agar may be used with trypsin as a plating medium instead of the inspissated serum where tissue bits are employed. With the addition of 1 per cent. of tryptophane it answers every purpose, whether the bacilli are planted with tissues or alone. It also serves to start multiplication of lepra bacilli that are contaminated at the time of plating. In the latter case the medium is "surface seeded" with an emulsion of the tissue juices in the same

manner as in preparing "streak" plates. The leprosy colonies in the thinner parts of the loop track are well separated and easily distinguished from those of the other species by their color and by their appearance only after two to five days.

In using the indirect method for the cultivation of *Bacillus leprœ* it is necessary only to provide the pieces of infected tissue with some bacterium capable of oxidizing the nucleo-proteids. A great variety of bacteria may be utilized for this purpose; among them may be mentioned *B. typhosus, paratyphosus, prodigiosus, pyocyaneus, dysenteriœ,* etc. In using the indirect method it is best not to employ a spore-bearing species as they are much harder to get rid of subsequently, though many of them answer the purpose equally well. Not infrequently a spore-bearing variety will be encountered in the tissues at the time of removal, but this is of little consequence as a second plating readily separates the contaminator. Putrefactive bacteria apparently do not interfere in the slightest degree with the multiplication of *Bacillus leprœ,* and when they hydrolize the tissue they serve as a valuable adjunct by converting the nucleo-proteid into suitable nutritives.

In using an agar medium it is well to leave out peptone and to titrate the reaction to 1.5 per cent. alkaline in order to prevent a too profuse growth of the associated bacteria; besides, an alkaline medium seems best adopted for the multiplication of the lepra bacilli. The method is to be recommended in all cases where the leprous tissue is contaminated with other microörganisms, as, for example, material from the naso-pharyngeal lesions or those on the exposed surfaces of the body.

As in the direct method, small bits of leprous tissue are placed upon the surface of the solidified alkaline agar and moistened with a thin suspension of some one of the bacterial species above mentioned. The plates are now incubated at 32° to 35° C. for two weeks, when they may be removed and portions of the softened tissue masses streaked over the surface of a second series of plates in order to obtain separated lepra bacilli colonies. It is necessary to use for the second plating either the trypsin-albumen medium or tryptophane agar.

Nutrient agar or any of the ordinary laboratory media will not serve as a transfer medium until the cultures have become accustomed to artificial conditions. So if the trypsin-albumen or tryptophane is not used it is necessary to keep the cultures growing with the associated bacteria for a much longer time, *i. e.*, until the lepra bacilli have acquired saprophytic properties. Recently isolated cultures require the dissociate products of tryptic digestion whether they have been previously produced in the medium or converted *in situ* by certain other bacteria. Glycerine added to the medium seems to enhance its nutritive value; at least the growth that results in its presence is more luxuriant.

Bacillus leprœ will also grow on the various blood agar media once they are accustomed to artificial conditions. The Novy-McNeal agar for the cultivation of trypanosomes gives a luxuriant growth of the organism if 2 per cent. glycerine has been added; without the glycerine, growth is very scant. Fluid media are not suited for the artificial cultivation of leprosy bacilli unless they are kept upon the surface. Like the tubercle bacillus they require abundant oxygen; those sinking beneath the surface cease to multiply but remain viable for months. Where it is desired to study their fermentative properties it is necessary first to grow the lepra organism on a solid medium and later to transfer parts of the growth to sterile floats (paper) that are carefully placed upon the surface of the fluid.

Ordinarily the growth of *Bacillus leprœ* is very moist and in this respect unlike that of *Bacillus tuberculosis,* except possibly the avian strain. Sometimes when the medium is devoid of water of condensation the growth is dry and occasionally wrinkled, though it is easily removed from the surface of the medium.

The chromogenic property of lepra cultures is a constant and characteristic feature for the rapidly growing stains. The color varies in degree of intensity depending upon the medium employed. If glycerinated agar (without peptone) is used, the colonies are faint lemon yellow, while on inspissated blood serum they are deep orange. It is noteworthy that the growth in the tissues and in the first dozen or so generations on artificial media is entirely without pigment.

CONCLUSIONS.

In the cultivation of *Bacillus leprœ* the initial multiplication outside the body cannot be obtained unless amino-acids are present in the medium. The amino-acids are believed to be essential nutritives for the initial growth of the organisms.

It has been demonstrated that the primary growth of the leprosy bacilli occurs only in the presence of the products of tryptic digestion.

Hence, putrefactive and other bacteria which are capable of splitting nucleo-proteids into their end acid products are, in consequence, of value in the isolation and cultivation of the leprosy bacilli. Amebæ are not necessary for securing the primary multiplication of the leprosy bacilli upon artificial media and are detrimental since they feed with avidity upon the bacilli themselves.

Two methods may be employed for recovering in culture *Bacillus leprœ* from the tissues. In one (the direct), tryptophane or a mixture of albumen and trypsin are employed with a culture medium; in the other (indirect), bacterial species capable of digesting the albumen constituent of the culture medium are introduced into the medium. In both, the end result is identical, since they both provide for the presence of the amino-acids in the medium, without which the primary multiplication of the leprosy bacilli cannot be secured.