METHODS OF CULTIVATION OF THE HANSEN BACILLUS

SLIDE CULTURE; HEMOLYSIS-TUBE CULTURE; DIRECT INOCULATION OF THE LIQUID MEDIUM[1]

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As a rule the difficulty of isolating a germ lies in finding the proper nutrient medium. When it is adapted to the medium, the germ can nearly always be transferred to various other media.

Ever since the time Hansen discovered the bacillus of leprosy many workers have tried to cultivate it, and many have thought they had succeeded. None of the many and varied cultures, however, has been proved by any means, including the immunological reactions characteristic of lepromin, to be the actual *Mycobacterium leprae.*

A review by McKinley (9) of work in this field showed that the cultures obtained by numerous workers needed confirmation, including the one isolated by himself with Soule, and there has been doubt whether any of the germs cultivated really had any relation to leprosy. Rotberg and Bechelli (10) have reviewed the subject more recently.

Souza-Araujo (12-14) has on numerous occasions isolated acid-fast bacilli from the nasal mucus and lepromas of patients, without however being able to demonstrate whether or not any of them was the Hansen bacillus. Recently Sister Marie-Suzanne (8) isolated an acid-fast bacillus to which the name *M. marianum* has been given, but the doubt about this bacillus is the same as in the previous cases. Floch (4) holds that *M. marianum* is neither "the" Hansen bacillus nor "a" Hansen bacillus; and Chaussinand and Viette (3), who regard it as a "paratuberculosis" bacillus, question the propriety of its use in therapy.

Hanks, among other things, has implanted bacilli from emulsified lepromas into various media supplemented with substances known to promote growth of acid-fast bacteria (5), and also has studied the fate of the bacilli when suspensions of the completely disintegrated tissue were incubated on standard solid or liquid media (6), asserting that no growth of bacilli could be proved.

Sato (11) has used a slide-culture method with various liquid media. He, too, was unable to obtain growth of the leprosy bacillus.

In this paper I report techniques for isolating a bacillus which I have been able to demonstrate is the authentic *M. leprae.* The process employed for this demonstration of authenticity will, however, be published sepa-

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[1] Work done at the Laboratory of Chemotherapy organized by the National Research Council of Brazil.

rately; that consists of the use of a medium in which subcultures can be obtained with the formation of globi.

A. ISOLATION OF THE HANSEN BACILLUS BY THE SLIDE CULTURE METHOD

In a previous paper (1) I have told of the slide-culture method which has been employed in this work. Under the conditions described—i. e., in a nutrient medium consisting of Kirchner's solution (7) to which 0.1 per cent agar and 20 per cent tyndalized normal human plasma were added[2]—there is proliferation of *M. leprae* with new formation of globi. This microculture, however, does not continue to grow when transferred to fresh medium of the same composition. Under these conditions proliferation of the bacilli depends on the tissue element of the leproma, which stimulates growth; when this tissue is used up proliferation ceases.

As previously reported (1), nontyndalised serum or plasma of a lepromatous patient facilitates proliferation, as it contains no antibodies. Therefore, for isolating the Hansen bacillus by the slide-culture method I have used a nutrient medium containing 20 per cent lepromatous serum or plasma. This serum or plasma may or may not be taken from the donor of the material containing the bacilli.

Suspension of the bacilli.—Leproma: The leproma is obtained with the greatest aseptic precautions. It is first cut up with scissors, and then triturated in a mortar with plasma or serum of a lepromatous patient. This suspension can be kept for several months in the refrigerator at a temperature of 4°C without loss of capability of proliferation on the part of the bacilli.

Nasal mucus: The nasal mucosa is curetted, and in a sterile hemolysis tube a suspension is made in the lepromatous plasma or serum. This suspension should be used at once.

Preparation of smears for cultivation.—A smear of the suspension of bacilli about 2 cm. long is made at one end of a slide measuring 1.0 x 7.6 cm. (an ordinary slide cut to 1 cm. width). The material is dropped onto the slide by means of a pasteur pipette and spread with a platinum loop to form a thin smear; if the smear is thick it easily comes loose. The smear is dried at 37.5°C, and then placed for 10 minutes in a 6 per cent solution of sulphuric acid. It is next washed twice in sterile distilled water for 1 to 2 minutes. After being placed in a tube containing Kirchner solution reinforced as described with agar and lepromatous serum or

[2] Formula for the Kirchner synthetic medium:

Na_2HPO_4	3.0 gm.
KH_2PO_4	4.0 gm.
Mg SO_4	0.6 gm.
Sodium citrate	2.5 gm.
Asparagine	5.0 gm.
Iron ammonium citrate	0.05 gm.
Glycerine	20 cc.
Distilled water	1,000 cc.

plasma, it is incubated at 37.5°C. The tube is closed with a cork and paraffined to prevent evaporation.

Several smears can be made at one time, some being used as controls after being fixed by heat and stained by the Ziehl-Neelsen method.

Bacilli for the preparation of the smears can also be obtained directly from the patient (leproma, nasal mucus or lymph).

RESULTS

To determine whether there is proliferation, the smears are taken out of the tubes, fixed by means of heat without washing—which would cause the smears to come loose—and stained. Comparison is made with duplicate smears not incubated in the medium.

Proliferation is observed from the 5th day of incubation, becoming more noticeable after the 15th day, when new formation of globi can be seen (see photomicrographs). After the 30th day there is turbidity of the medium, due to escape of proliferated bacilli from the smear culture. Tubes containing only liquid medium show no growth, proving that the culture originates in the smear and not in the serum or plasma of the patient.

Subcultures.—Subcultures can be obtained in series by transfer to a medium consisting of Kirchner's solution with 20 per cent tyndalised normal human serum. The serum or plasma of the patient is no longer necessary to stimulate proliferation. In these free-growing subcultures there is no formation of globi.

Pseudo-yeast.—As has been pointed out previously (1, 2), the medium after incubation often contains a pseudo-yeast which preexists in the material inoculated and proliferates in the smear culture. However, when the smear remains for longer than 10 minutes in the sulphuric-acid sterilizing solution, growth of the pseudo-yeast is less frequent. When it occurs, the growth of this organism exceeds that of the Hansen bacillus. The levuriform germ can be isolated by successive transfers to Sabouraud's medium at 37.5°C or at room temperature.

B. ISOLATION OF THE HANSEN BACILLUS BY CULTURE IN THE HEMOLYSIS TUBE

In this variant of technique the same nutrient medium is used as in the slide culture method, but the hemolysis tube is substituted for the slide.

The smear is made—with material obtained, as described above, from a suspension of leproma or directly from the nasal mucus, a leproma, or lymph of the patient—in such a way as to form a film on the inner wall of the tube. The part near the mouth of the tube is left free, so as to permit of sterilization of that part by heat without affecting the smear. The tube is left open at 37.5°C until the smear is completely dried. To leave the dried tube for 30 days at room temperature before proceeding

with the further manipulation described does not prevent proliferation of the bacilli.

To sterilize this smear, the tube is filled nearly to the top with the 6 per cent sulphuric acid solution. After 10 minutes this is poured off and the tube is filled, twice, for 2 minutes each time, with sterile distilled water. Then the Kirchner-agar-lepromatous serum (or plasma) medium is poured in to cover the smear. The tube is closed with a sterile cork and treated with paraffin to prevent evaporation.

After 30 days incubation at 37.5°C, when proliferation is abundant, transfer is made to the Kirchner-tyndalised normal serum medium. Neither agar nor lepromatous plasma is now necessary.

The advantage of using the hemolysis tube is that the bacillus-containing material can be obtained directly from the patient, and that sterilization is facilitated by being effected in the tube itself.

C. DIRECT INOCULATION OF THE LIQUID MEDIUM

Another culture method employed was direct inoculation of the Kirchner-agar-lepromatous serum medium with the leproma suspension prepared aseptically. The suspension was made with 4,000 units of penicillin per cubic centimeter of medium as the bacteriostatic agent. In this case the sterilization by the sulphuric acid solution was not necessary.

The turbidity of the medium observed by the 20th day shows that there is proliferation. In the same way as already indicated, subcultures can be obtained.

ISOLATIONS MADE

Five isolations of the Hansen bacillus have been effected to the time of writing, each designated by the name of the patient concerned.

By the slide-culture method, two isolations: in May 1954, the Albertina strain, from a leproma; on June 9, 1954, the W. F. strain, from the nasal mucosa (see photomicrographs).

By the hemolysis tube method, two isolations: on January 14, 1955, the Luiz strain, from a leproma; on April 25, 1955, the Quepard strain, from the nasal mucosa.

By direct inoculation of the liquid medium, one isolation: on April 29, 1955, the Alfredo strain, from a leproma.

These five successful cultivations are from a series of some 17 attempts, made with material from a total of 12 patients. The failure of growth in the other cases might have been due to the presence of specific antibodies in the lepromatous serum used in the culture medium. This explanation might be true especially in the case of patients with a tendency to the "borderline" form of the disease.

All five of the cultivated strains grow in new subcultures when transferred to the Kirchner-normal serum medium. To the time of the writing of this paper the W. F. strain has been cultivated in 15 successive trans-

fers. These cultivations were made in attempts to obtain typical globi in an adequate medium, and also to obtain material for animal inoculations for the production of experimental infection. The four other strains have been grown in series varying from 3 to 8 successive transfers. They are being kept at 37.5°C in this liquid medium. Old cultures have a straw-yellow color, and a smell of ammonia.

All these strains, when cultivated in Kirchner-serum medium and stained by the Ziehl-Neelsen method, are seen to consist of short, acid-resistant bacilli containing purple granules. More rarely, the bacilli are long and granulated. Sometimes under the microscope are seen large spheric masses in which the bacilli are distributed at random. However, I have been able to devise a medium (to be the subject of another publication) in which the bacilli can be seen to multiply by longitudinal division, forming rows of 4 to 6 bacilli. These groups develop later into typical globi.

In this medium the rate of growth of the Hansen bacillus is about the same as that of the Koch bacillus. When the medium contains no tyndalized normal human serum, the bacilli undergo lysis. Although usually none of the strains grows in Petragnani's medium, almost invisible colonies may sometimes be seen after a few months.

SUMMARY

By the method of slide culture in a nutrient medium consisting of Kirchner solution with 0.1 per cent agar and 20 per cent lepromatous plasma or serum, a culture of *M. leprae* can be obtained by incubation at 37.5°C. Under these conditions, by comparison of the control smears with those incubated in the culture medium, we can be certain that we are dealing with the Hansen bacillus because of the presence of newly-formed globi. After incubation for 30 days the nutrient medium becomes turbid, due to bacilli which have proliferated in the smear and fallen into the medium.

By transfer to a medium consisting of Kirchner solution with 20 per cent tyndalised normal human serum, we can obtain an abundant culture of free-growing *M. leprae* which can be subcultivated in series. In this medium, however, there is no further formation of globi. We get colonies of acid-fast bacilli in which the bacilli are scattered at random.

A variant of this method consists in substituting the slide by a hemolysis tube on the wall of which the smear is made, using the same medium as in the former method. When the culture in the hemolysis tube is abundant it can be transferred to the medium in which tyndalised normal serum is used.

In both methods the smear can be made from a suspension of leproma or from nasal mucus, or directly from the leproma, nasal mucus or lymph applied to the slide or hemolysis tube.

The slide culture permits of microscopic examination of the growth,

but culture in the hemolysis tube has the advantage of being made with larger quantities of the material inoculated and, in addition, sterilizing of any contaminants present is effected in the tube itself. As the dry smear can remain in the tube at room temperature for at least 30 days without preventing proliferation, it can be sent great distances to be cultivated.

When the leproma is handled with necessary aseptic care, penicillin (4,000 units per cc.) can be employed as the bacteriostatic agent in the leproma-suspension preparation instead of the sulphuric acid solution. This makes the method extremely easy, because the nutrient medium can be inoculated directly without previous sterilization of the leproma suspension.

RESUMEN

Descríbense técnicas con las que se ha cultivado el bacilo de Hansen en medios líquidos: cultivo en placas, cultivos en tubos de hemólisis e inoculación directa en el medio líquido. El inóculo es una suspensión de leproma triturado, aunque también pueden usarse materias tomadas directamente de la mucosa nasal, un leproma o linfa.

El medio primario, en todos los casos, es la solución sintética de Kirchner, a la que se agregan 0.1 por ciento de agar y 20 por ciento de suero o plasma procedente de un enfermo lepromatoso. Se obtienen subcultivos en el medio de Kirchner con 20 por ciento de suero humano normal tyndalizado.

El método de los cultivos en placa posee la ventaja de que pùeden hacerse, después de la incubación, exámenes microscópicos para comparación con los frotes usados de testigos. Al proliferar los bacilos en la placa, obsérvase neoformación de masas bacilares esféricas, o globos (*v.* microfotografías), lo cual identifica las colonias como bacilos de Hansen. En los subcultivos no se forman globos. Se mencionan los cinco aislamientos verificados.

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Description of Plate

Plate (2)

The four pictures here shown are of the preparations from Case W. F. (see text), showing the appearance of a slide smear after incubation as compared with a control smear.

Fig. 1. Control smear from the nasal mucosa.

Fig. 2. Detail of the preparation shown in Fig. 1, higher magnification.

Fig. 3. Slide culture after incubation for 15 days. Note the new formation of spherical masses of bacilli (globi).

Fig. 4. Detail of the preparation shown in Fig. 3, higher magnification.

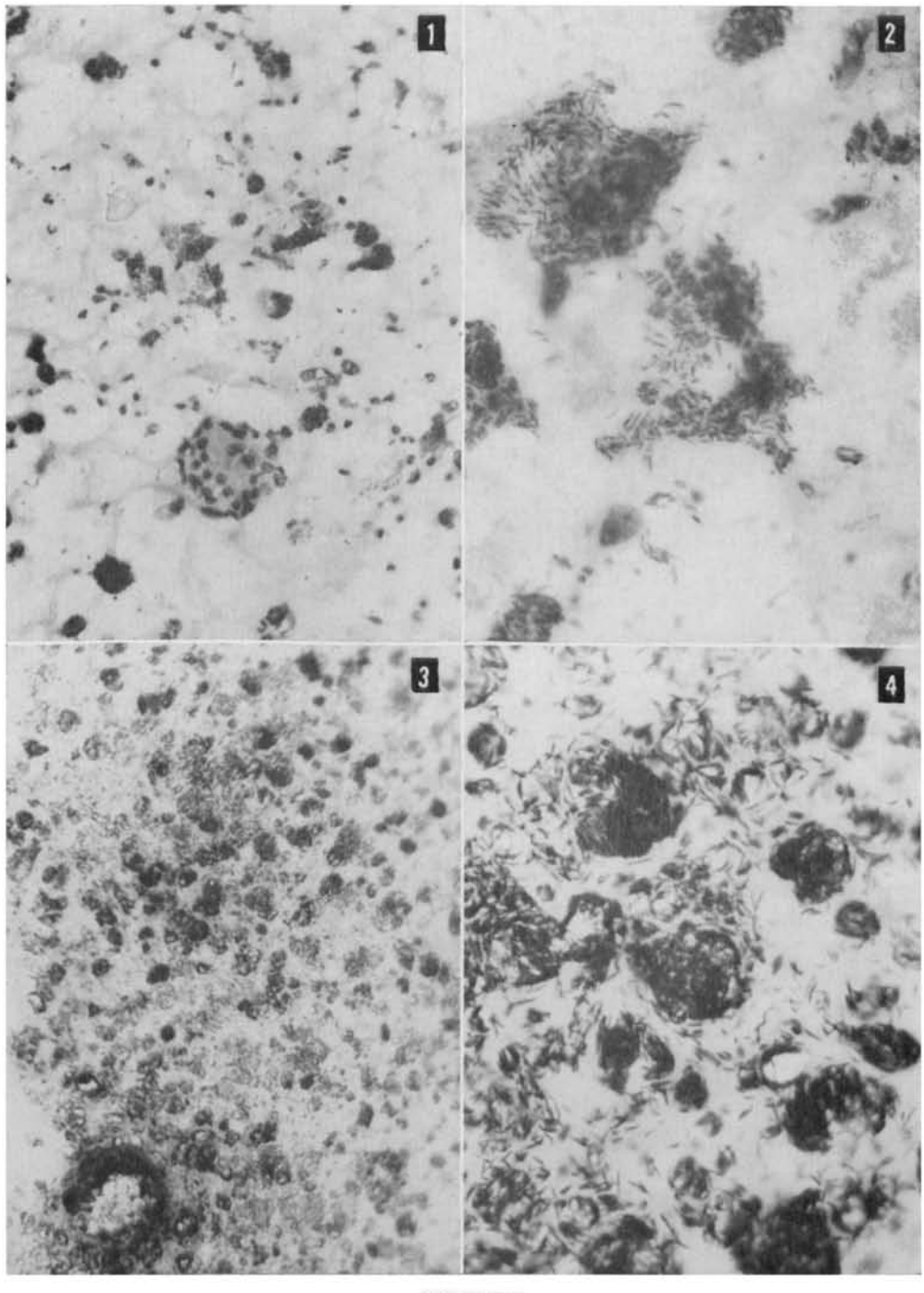

PLATE 2