



THE CULTIVATION OF THE LEPROSY BACILLUS
PRELIMINARY COMMUNICATION

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It is not at all certain that the cultivation of the leprosy bacillus has been accomplished heretofore, for the various claims which have been made for the isolation of that organism have not been satisfactorily supported. That this is true is evident from the literature on the subject, which has been dealt with most thoroughly by Jadassohn, of Breslau, in the Handbook of Pathogenic Organisms, of Kolle, Kraus and Uhlenhut. The whole history of the subject shows that definite conclusions cannot be drawn from isolated findings, but I consider it desirable to record here the observations which I have recently made on the matter.

My work on the cultivation of acid-fast bacteria extends over a long period, thirty-two years. Three years ago Dr. Lie, the director of the leper asylum in Bergen, Norway, worked with me here in Vienna for six weeks, studying the problem of culturing the Hansen bacillus. The first difficulty in this work lies in the fact that this bacillus will not grow at all upon the usual culture media. Secondly, it is often found living in symbiosis with other bacteria in the human lesion; these accompanying bacteria are always present in extirpated nodules and naturally overwhelm the poorly-growing leprosy bacilli. In order to avoid the latter difficulty I have not worked with tissue, but with the blood of persons infected with leprosy.

EARLIER EXPERIMENTS

In the earlier experiments the blood specimens were dealt with in essentially the same manner as those cultured for the tubercle bacillus. This included treatment with sulphuric acid by the method which I have described (1923).

The blood was obtained uncontaminated by means of collecting ampules of the "Kovacs" type, in which there is 3 cc. of 10 per cent sodium citrate to prevent coagulation. It was first freed of hemoglobin by being placed in a centrifuge tube of 40 cc. capacity, treated twice with large amounts of sterile distilled water, and centrifuged. After the second or third washing there remained a whitish, hemoglobin-free sediment. This was then treated with 1 cc. of 15 per cent sulphuric acid for five minutes, with frequent shaking, after which 30 cc. of sterile distilled water was added, with continued shaking, and the suspension was rapidly centrifuged once more. This last process was repeated twice again in order to remove the sulphuric acid more completely, so that after this last washing the sediment was only slightly acid. The entire sediment was taken up with a pipette and used to inoculate the surface of the media. The glycerin-egg media used I have previously described in other publications.¹

RESULTS

In the first generation of growth we found colonies of microscopic size which consisted only of thin acid-fast bacilli. Upon re-cultivation the subplants, after four weeks, showed only a very few acid-fast bacilli. It was clear that these had not increased by growth but rather were remnants of the inoculated material from the first generation. In the third series of cultures acid-fast bacilli were no longer to be found. Thus these experiments came to no conclusion except to demonstrate that our usual culture media were entirely unsuitable for the purpose. Blood received later from Riga and Granada, as well as from Dr. Sundaram of Kilpauk, Madras, all similarly gave only short-lived cultures.

FURTHER EXPERIMENTS

Five blood samples were received, in October, 1933, from Dr. M. H. Soule, of the University of Michigan, who at that time was working at the Culsion Leper Colony in the Philippines under the auspices of the Leonard Wood Memorial. In spite of the long trip from Culsion to Vienna the blood samples, which were shipped in Kovacs tubes, arrived in good condition, only slightly contaminated, and were immediately cultured.

Because of my earlier experience I now turned from solid to liquid culture media, and employed several new ones. I had already tried media made with vegetable materials—asparagus, carrots, tomatoes, potatoes, etc.—but all these attempts failed. Finally, I made a simple fish broth, soaking 250 grams of

¹ See this JOURNAL 1 (1933) 39.

carefully ground cabeljau in two liters of distilled water for 24 hours and then cooking it in the autoclave under two atmospheres for an hour. This was then filtered clear, glycerinated (2 per cent), made alkaline (pH 7.8—8.0), and put into flasks in 100 cc. lots.

Each blood specimen was divided in two equal parts, de-hemoglobinized, and the sediments dealt with in two different ways: (1) One set of five sediments was treated with 15 per cent sulphuric acid to kill off the contaminating organisms, washed twice with distilled water, and planted in flasks of the fish-broth medium. (2) The other set of five sediments was not acid-treated, but was simply washed twice with distilled water and then distributed to other flasks of the medium.

RESULTS

Non-acidified material.—After four weeks incubation two of the flasks planted with the material which had not been treated with sulphuric acid (Nos. 1 and 5) showed marked increase of leprosy bacilli. In the microscopic preparations one could see unstained, fine detritus which by its arrangement in stripes and lines gave rise to the suspicion that these non-acid fast forms were part of a developmental cycle of the leprosy bacillus. However, intermixed with these one also saw entire clusters, sometimes star-shaped and sometimes in bundles, of small acid-fast bacilli which were of an especially bright red color after staining by the Ziehl-Neelsen method.

In the other flasks inoculated with the same material the leprosy bacilli increased in spite of the fact that there were also present various other forms of bacteria, including spore-bearers and in one case a sarcina. It thus appeared that the leprosy bacillus is not so sensitive to mixed infection as is the tubercle bacillus.

Acid-treated material.—All of the flasks which had been planted with the acid-treated material were found to have rich growths of leprosy bacilli, but some of these were also contaminated (Nos. 2, 3 and 4). The period of time that the acid had been allowed to act on the sediment was only five minutes, for I had assumed that the leprosy bacillus would be less resistant to it than the tubercle bacillus.

From the two flasks which had remained uncontaminated (Nos. 1 and 5) growth was transferred to tubes of a solid medium. This was composed of the usual glycerin-egg mixture to which, however, fish-broth had been added in the proportion of 100 cc. to 1200 cc. After four months very small colonies of macroscopic size and whitish color had developed. These colonies were not as hard as

are those of the tubercle bacillus, but they formed wart-like growths which otherwise were similar to them, and they were assumed to be that organism. However, there were also present very small colonies with a light-yellow tint, which brought to mind the supposed leprosy-bacillus cultures that I have had the opportunity to see; these were all pigment-producers whose colors varied from yellow to bright red, just as we so often see in the acid-fast bacilli found free in nature.

Further cultivation of these colonies upon the fish-broth-egg medium proved the correctness of our supposition. From the white colonies we succeeded in cultivating organisms which had all the characteristics of true mammalian tubercle bacilli. From the small yellowish colonies, which were moist, there appeared on sub-culture new small yellowish colonies which formed an extraordinarily delicate yellowish-white surface upon the culture slant. After two months growth the cultures showed the picture of microscopic "seed-rows" or the form of "strings of pearls." However, in this stage the acid-fast properties had not yet been fully developed, though one could always observe single leprosy bacilli and sometimes even small groups. After four weeks observation of these cultures it was seen that the seed-row appearance had almost disappeared and had been replaced by long, thin bacilli which were somewhat longer than true leprosy bacilli. In this case, also, the Ziehl-Neelsen stain gave a brighter red color to the bacilli than is found in tubercle bacilli, and one had the impression that there was a colorless interstitial substance between the bacilli.

In the following generation in these cases (Nos. 1 and 5) the organisms grew on the solid medium, as in the flasks, most rapidly on the fish-broth-egg medium. After eight weeks there was a definite moist yellowish growth, though upon my original glycerin-egg medium none was visible. After another eight weeks the growth contained large masses of acid-fast leprosy bacilli. One also saw, however, non-acid-fast bacilli which morphologically were identical with the leprosy bacilli.

These strains produce acidity. My media that contain the fish-water³ have a pH of 7.8, at which concentration the malachite-green

³ The composition of the medium without fish-water is given in my previous article in this JOURNAL.

takes a yellowish color. As soon as acid is added the blue-green color reappears, this serving as an indicator to warn one if the sediment is not carefully washed. It has been observed that after five months growth the leprosy bacillus colonies become greenish-blue, in contrast to the colonies of the Koch bacillus which retain their grayish color.

Turning now to the flasks containing growths in fish-water-asparagin solution, the original pH of which had been 7.8-8.0, I was much surprised to find that the pH had gone down, the concentrations after 4, 5 and 6 months being 4.5, 4.2 and 4.0, respectively. There is no doubt that the leprosy bacillus is a strong producer of acidity, in contrast with a series of other acid-fast germs, which produce alkaline reactions.

With the pure cultures in hand animal experiments have been undertaken, but these are not yet complete for the animals must be kept under observation for at least a year. Certain cultures caused typical tuberculosis in guinea-pigs; the others were inoffensive and produced no allergy to human tuberculin.

CONCLUSIONS

1. Cultivation of the leprosy bacillus has been successfully accomplished by means of my sulphuric-acid method on an egg medium to which fish-broth has been added.
2. The statements of Lie and others that leprosy bacilli are found in the blood have been verified both by direct smear and by culture. In two out of five cases a pure culture was obtained from the blood. In two cases tubercle bacilli were also found in the blood, along with the leprosy bacilli.
3. The growth of this leprosy bacillus is very slow, taking up to six months to form macroscopic colonies on my fish-egg medium.
4. These leprosy strains have not shown evidence of pathogenicity in guinea-pigs observed for six months.
5. They are characterized by a strong capacity of acid production, both on the egg medium and in the fish-asparagin solution.