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Studies with Chick Embryo Tissue in Cultivation of B. Leprae.

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Since the discovery by Hansen of the acid-fast bacillus in the lesion of human leprosy, many investigators who have attempted its cultivation have considered their "culture" the authentic one. Recently Soule and McKinley[1] in their attempts at cultivation of *B. leprae* prove no exception to this rule. In an earlier publication[2], they claim complete success with the CO_2, O_2 method of Wherry[3] regardless of the nutritives employed. McKinley and Verder[4] later claim that they have cultivated *B. leprae* in 5 days in chick embryo tissue suspended in Tyrode's solution under ordinary atmospheric conditions as well as CO_2 and O_2.

At the suggestion of Dr. Duval I have undertaken to repeat the work of McKinley and Verder. This paper reports results obtained upon that part of the work in which the chick embryo tissue is concerned.

The leprous tissue employed was the subcutaneous nodules and material from the socalled leprous abscess of human lepers, kindly supplied us through the courtesy of Dr. Denney of Carville, La. All the material was extremely rich in acid-fast bacilli as was evidenced upon microscopic examination of stained tissue sections and smear preparations. The Hansen microorganism of leprosy appeared chiefly in densely packed masses (globi) and small packets.

The leprous tissue was first cut into small pieces aseptically, then placed in a mortar and emulsified. A part of the emulsion was treated with a 3% NaOH solution for one hour, and immediately afterward was centrifugally washed several times in sterile Ringer's fluid. Leprous material which was not contaminated was used without treating it with NaOH. In addition, the purported culture of *B. leprae* in minced chick and Tyrode's solution which was sent to Dr. Duval by Dr. McKinley, was used in parallel experiments. Both liquid and solid media (plain neutral agar) containing the emulsified chick tissue were employed in the experiments. Ten-day-old chick embryos which had been aseptically removed from their

[1] Soule and McKinley, *Am. J. Trop. Med.*, 1932, **12**, 1.

[2] Soule and McKinley, *J. Am. Med. Assn.*, 1932, **98**, 361.

[3] Wherry, *J. Infec. Dis.*, 1930, **46**, 263.

[4] McKinley and Verder, PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 659.

shell and shell contents formed the basis of all culture media. The embryos were minced and suspended in Tyrode's solution, Ringer's fluid and adult chicken serum respectively. The proportion of chick embryo tissue to fluid medium was approximately 1 to 2.

The various liquid media were inoculated with the leprous tissue emulsion. In the instance of the agar medium, the chick emulsion was spread over the slanted surface and the leprous inoculum placed or rubbed into this with a sterile platinum loop. A set of the inoculated media were incubated at 37°C. under ordinary atmospheric conditions and another set was incubated in Novy jars under CO_2, O_2 tension. The percentage mixture was 10% CO_2 and 40% O_2. After periods of 5, 7 and 30 days, the tubes incubated under ordinary atmospheric conditions were examined microscopically to determine whether proliferation of *B. leprae* had occurred. For this purpose smear-preparations were made and stained by the Ziehl-Neelsen method. The inoculated culture tubes under gaseous tension were not examined until one month after incubation.

In the determination of *B. leprae* proliferation in culture, we used the only index or guide available, namely, stained smear preparations from the original leprous emulsion before its inoculation to culture media. It is realized that this method at best is no reliable criterion of multiplication because no 2 smear-preparations from either the leprous inoculum or the "culture" will reveal the same microscopic picture, since with any 2 such preparations, one may show many times more acid-fast bacilli than the other. Furthermore, in making a smear-preparation from a "culture", one is as likely as not to take up on the platinum loop a small bit of the originally planted leprous tissue which, when smeared over the glass slide and stained, will reveal enormous numbers of acid-fast bacilli as compared to the index slide. This likely error in judging of microscopic growth in culture is all the more appreciated if we realize as Duval[5] has pointed out, that *B. leprae* multiplies in autolysing leprous tissue. One must be extremely careful not to mistake "proliferation" in tissue transplants for true *in vitro* growth of *B. leprae*. Multiplication is determined not so much upon what seems to be an increase in numbers of acid-fast bacilli as on the change in their morphology and tinctorial reaction. The new forms are longer, thicker and possess definite bipolar granules, while the original bacilli are shorter, thinner and apparently fragmented. As a rule, the new bacilli are not arranged in groups of parallel rods, nor do they occur in pack-

[5] Duval, C. W., *J. Exp. Med.*, 1910, **12**, 649.

ets or globi as they commonly do in the lesion of human leprosy. With respect to tinctorial differences, the new bacilli are distinctly more acid-fast, *i. e.,* they stain more intensely and retain the carbol-fuchsin after longer attempts at decolorization. The entire outline of the new bacilli is sharply discerned, whereas in the case of the old original forms, it is seen with difficulty.

Lack of growth of *B. leprae in vitro* is evidenced by the fact that the bacilli remain as they were originally in the leprous tissue before culturing. They are, in comparison to the new forms, thin, indifferently stained, fragmented rods of varying lengths that lie more or less close together in parallel arrangement or they are concentrically aligned in masses known as "globi".

Morphological signs of retrograde changes and loss of viability occur for *B. leprae* in certain cultural environments. Here the bacilli, as repeatedly observed in our cultural studies, are weakly acid-fast or only partly resistant to the ordinary decolorizing agents. The "rods" lose their cell outline and the chromatin becomes more fragmented and granular. The "globi", though retaining their acid-fastness, appear as homogeneously pink, finely granular masses after treatment by the Ziehl-Neelsen method.

Comments. All cultures that had been incubated under ordinary atmospheric conditions, were examined microscopically at varying intervals over a period of one month. Particular attention was paid to the claim of McKinley and Verder that growth of *B. leprae* occurs in 5 days upon chick-embryo emulsed in Tyrode's solution. The microscopic criteria of growth used by us are given in the text.

No positive evidence of *B. leprae* proliferation was noted at any time in chick-embryo emulsified in Tyrode's solution for the various inoculums used, including the purported culture received from Dr. McKinley. On the contrary, many of the acid-fast bacilli of Hansen in this medium showed definite signs of degeneration and loss of viability after four weeks incubation at 37°C. Furthermore, negative cultural results were obtained with the inoculated chick-embryo emulsion under both ordinary atmospheric and CO_2, O_2 conditions.

In the light of our experiments chick-embryo tissue emulsified in Tyrode's solution is *per se* valueless as a nutritive in the *in vitro* cultivation of *B. leprae.*