BACTERIOLOGY OF LEPROSY[1]
V. FURTHER ISOLATION RESULTS

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In a previous communication (2) a description of an acid-fast organism isolated from human and rat leprous lesions was reported.[2] Embryonic chick tissue-cultures were used as the isolation medium. Later reports (3, 4, 5) have dealt with the influence of various environmental factors on the tinctorial characteristics of the organisms that were isolated.

The present communication deals with results obtained in work done at the United States Marine Hospital, Carville, Louisiana. The same media as reported in the first paper of the series were employed, namely, embryonic chick tissue cultures, minced chick embryo medium, and glycerin veal agar slants. In addition, the above methods were paralleled by the procedure recommended by McKinley and Soule (1), employing glycerin veal agar slants kept under a gaseous environment of 40 percent oxygen, 10 percent carbon dioxide and 50 percent nitrogen.

The purpose of the work was to determine whether or not the same organism could be isolated repeatedly by the method outlined in the first report of this series. A satisfactory experimental animal for human leprosy is not available, but if the same organism can be isolated repeatedly in pure culture, that may be indirect evidence that the cultured form is the true etiological agent of leprosy.

There is considerable doubt in the minds of many workers that a satisfactory experimental animal would determine whether or not the isolated organism is the true agent of the disease. It is known that if rat granuloma is kept in the laboratory for several weeks and then injected into young normal rats, it is unable to produce rat leprosy. In other words, the material must be injected into new

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[2]The work here referred to was carried out at the Leprosy Investigation Station, U. S. Public Health Service, Honolulu, Hawaii.

rats shortly after being removed from a diseased animal. If rat leprosy cannot be produced from old rat granuloma, what results could be expected from the injection of material removed from laboratory cultures after several generations of growth?

EXPERIMENTAL

The work here reported was limited to the three types of culture media mentioned above. They were (*a*) glycerin veal harmone agar, (*b*) minced chick embryo medium, and (*c*) chick tissue cultures. The media were prepared with great care both as to composition and hydrogen ion concentration.

Tissue cultures.—The medium consisted of chick tissue fragments, guinea pig plasma, Tyrode solution and dilute embryonic fluid contained in 3.5 cm. Carrel flasks. The tissues were obtained from 7 to 10 day old chick embryos. Tyrode solution was prepared according to formula, plus the addition of 20 cc. of plain broth to each liter of solution, this being used as a nutrient to facilitate the detection of contaminants in the finished solution. The preparation was filtered through a Berkefeld filter and the filtrate distributed into test tubes. All tubes were incubated at 37° C. for 48 hours before use, and those that showed any turbidity were discarded. The guinea pig plasma was collected by cardiac puncture, the blood being mixed with sterile 1:1000 heparin solution in the proportion 10 to 1 and immediately centrifugalized in centrifuge cups previously filled with ice water. The heparinized plasma was removed with a pipette, expelled into a test tube cooled in ice water, and was kept in ice water until used. Embryonic fluid was prepared from minced chick embryos diluted seven times with Tyrode solution and centrifugalized. The supernatant fluid was distributed into test tubes in 2.5 cc. amounts. Sterility was tested by planting 0.5 cc. from each tube into plain broth and incubating at 37° C. for 5 days.

Minced embryo medium.—The medium was prepared with 1 part of minced chick embryos (7 to 12 days) and 5 parts of Tyrode solution. The embryos were decapitated, minced in a tissue grinder, and mixed with Tyrode solution. Amounts of about 3 cc. of the suspended tissue were measured into test tubes, after which the medium was ready for use. The heads were removed because the pigment granules of the eyes often led to confusion when smears were made to determine the sterility of the medium or to note presence or absence of growth of the inoculated organisms.

Material—Nodules were removed from seven patients by cautery and from others by incising the skin and carefully dissecting

the nodule. All nodules selected were nonulcerating, to eliminate the possibility of any external contamination. Strict antiseptic and aseptic precautions were observed. A total of eleven nodules was used.

All nodules were ground with a heavy glass rod in a sterile, heavy-walled test tube containing bits of broken glass. The neck of the tube was protected from external contamination by wrapping a strip of cotton and gauze padding around the rod before inserting it into the tube. The ground nodule was diluted with Tyrode solution to form a heavy suspension and pipetted into a sterile test tube. After the coarse particles settled out the supernatant fluid was used for cultivation experiments. All tubes of culture media were inoculated with two to three drops of the nodule suspension by means of a Pasteur pipette.

The fragments of chick tissue (from 0.5 to 1.0 mm. in diameter) were immersed for a few minutes in a dilute Tyrode suspension of the organisms, removed with a Pasteur pipette, and embedded in plasma previously diluted with 3 parts of Tyrode solution in Carrel flasks. The plasma was heavily inoculated with tissue fragments. After the coagulated plasma had been washed with Tyrode solution, the embryonic fluid was added. The flasks were stoppered with rubber caps and incubated at 37° C. The tissue fragments grew luxuriantly, visible evidence of growth occurring in every case between the fifth and tenth day.

The glycerin veal agar slants and the minced embryo tubes were inoculated and divided into two portions. One portion of each was placed in closed jars and the air removed until a vacuum of 28 inches was reached. The air was then replaced with a gaseous mixture composed of 40 percent oxygen, 10 percent carbon dioxide and 50 percent nitrogen, according to the method of McKinley and Soule. This procedure was repeated to compensate for the 2 inches of air pressure still remaining in the jars after the first evacuation. The tubes comprising the other portion were stoppered with paraffined corks under normal atmospheric gases. All tubes were incubated at 37° C. until visible evidence of growth occurred.

RESULTS

The results of cultivation experiments are given in Table 1, from which it is seen that growth was obtained from every nodule cultured. Positive growth occurred in 190 tubes and flasks out of the total of 311 inoculated. The use of tissue cultures was discontinued after the first five cultivations because of positive results with the

TABLE 1.—*Results of cultivation experiments.*

Experiment	Patient	Media used									
		Glycerin veal hormone agar				Minced chick embryo				Tissue cultures	
		Normal gaseous environment		Artificial gaseous environment		Normal gaseous environment		Artificial gaseous environment		Normal gaseous environment	
		Number of tubes inoculated	Number of tubes positive	Number of tubes inoculated	Number of tubes positive	Number of tubes inoculated	Number of tubes positive	Number of tubes inoculated	Number of tubes positive	Number of tubes inoculated	Number of tubes positive
A	A. O.	5	0	5	0	5	0	5	0	7	7
B	G. D.	5	3	5	3	5	5	5	5	7	6
C	J. S.	10	4	5	3	—	—	—	—	8	2
D	G. D.	5	0	10	3	—	—	—	—	7	3
E	S. M.	5	3	10	4	5	5	5	5	7	3
F	J. G.	15	15	10	10	6	6	6	6	—	—
H	J. G.	10	7	5	3	6	5	6	5	—	—
K	J. G.	10	2	10	9	8	8	7	7	—	—
L	R. W.	10	2	9	0	8	8	8	7	—	—
M	R. W.	9	3	10	8	6	6	6	6	—	—
N	E. M.	9	2	—	—	6	1	—	—	—	—
TOTALS		93	41	79	43	55	44	48	41	36	21

other media, this resulting in a great saving of time and labor. Many of the glycerin veal agar slants showed no visible evidence of growth, but when some of the material was removed from the butt of the tubes and transferred to fresh glycerin veal agar slants or to minced chick embryo medium, growth resulted in almost every case.

Growth was obtained in the artificial gaseous environment employed by McKinley and Soule, but growth equally as good was obtained under natural environment.

In no case was a culture of a pure acid-fast organism obtained. This applied to both the normal and the artificial gaseous environments. Also, none of the positive cultures on glycerin veal agar showed the presence of multiplying acid-resistant organisms. However, when some of the growth on the glycerin veal agar slants was transferred to minced embryo medium or to embryonic chick tissue cultures, both acid-fast and acid-sensitive organisms occurred.

Morphologically and tinctorially the organisms isolated from the various nodules appear to be alike. All of the strains showed the same characteristics of a mixture of acid-fast and nonacid-fast rods in cultures with tissue, and nonacid-fast organisms on the usual laboratory media. Young cultures showed a preponderance of acid-fast forms, but as the cultures aged the numbers of these forms became progressively fewer. On transferring some of the growth from an old culture to fresh medium the same picture was repeated.

The colonial characteristics of the organism varied from culture to culture on the same medium and also from one type of medium to another. For this reason no reliance was placed on the results of fermentation reactions. Visible growth on the glycerin veal agar slants showed a pink or light red pigment, but when some of the growth was transferred to fresh medium the pigment disappeared.

The organism isolated from patients in Honolulu possesses an orange pigment. Recent work has shown that by transferring this organism to a medium composed of minced rabbit lung suspended in Tyrode solution the pigment disappears, and all attempts to convert the nonpigmented form to the pigmented one have failed. The Carville strains were pigmented when isolated but apparently lost their ability to produce color when subcultured. In view of the fact that the pigmented organism isolated in Honolulu was converted into a permanently nonpigmented form, it is not believed that the presence or absence of pigment would rule out the possibility of the two organisms being the same.

No growth of pyogenic or other common bacteria occurred in

any of the tubes. This would indicate that the organisms cultivated did not result from imperfect sterilization of the skin. If the cultured forms are not the true organism of leprosy, then they must be present in the nodules in association with the etiological agent of the disease. Direct smear examinations from nodular material, however, failed to show the presence of organisms other than true acid-fast rods.

SUMMARY

Results obtained from cultivation experiments employing glycerin veal agar slants, minced chick embryo medium, and chick tissue cultures are reported. A total of eleven nodules from seven patients were used in the experiments, and 311 tubes and flasks were inoculated, from which 190 successful cultures were obtained.

The cultivated organisms were, in every case, acid-sensitive on glycerin veal agar slants. In minced embryo medium and in chick tissue cultures, however, a mixture of acid-sensitive and acid-resistant forms appeared. Young cultures showed a preponderance of acid-fast forms in the tissue media. As the cultures aged the numbers of acid-resistant organisms became progressively less. On transferring some of the growth from an old culture to fresh tissue media the same picture was repeated.

Morphologically and tinctorially the organisms appear to be similar to those previously isolated from patients in Honolulu, and their characteristics as regards acid-fastness are similar. It is believed that the results represent a confirmation of those previously reported in the first paper of the present series.

REFERENCES

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