

THE NEUTRALIZATION OR DESTRUCTION OF DIPHThERIA TOXIN BY TISSUE*

By AUGUSTUS WADSWORTH, M.D., AND ELLA N. HOPPE

(From the Division of Laboratories and Research, New York State Department of Health, Branch Laboratory, New York, N. Y.)

(Received for publication, March 21, 1931)

In the present stage of our knowledge the most direct approach to the study of the action of bacterial toxins in the tissues and the reactions of different tissues to their poisons appears to be the determination of the changes that take place when the toxin and the tissue cells are brought into contact under experimental conditions. Earlier studies (1) record the absence of any action between diphtheria toxin and the leucocytes or brain tissue of either the dog or the guinea pig; also the absence of reaction between these leucocytes and tetanus toxin, although the observations of Wassermann and Takaki (2) that brain tissue neutralizes tetanus toxin were confirmed. The development of the technic of tissue culture suggested the resumption of experimental study of this field. Meanwhile the literature has recorded the reports of other observers which intimate a selective action of these and of other bacterial toxins on the cells of tissue cultures.

Cultures of various tissues, chiefly from chicken embryos but also from some of the common laboratory animals, such as the guinea pig, rabbit, rat, and mouse, have been studied since Levaditi (3) noted the insusceptibility to diphtheria toxin of the cultures from the hematopoietic system in the dilutions which he used. This is in conformity with our observations on leucocytes but it would appear from his experiments with cultures of heart tissue that these were affected by diphtheria toxin and that they were protected from this action by the presence of antitoxin.

Similarly, Burrows and Suzuki (4) record the action of diphtheria toxin on the growth and vitality of tissue cultures, confirming the observations of Levaditi, as have several more recent investigators. Mendeléeff (5) tested cultures of heart tissue of the guinea pig. Kimura and Ishii (6) found that fibroblasts were not susceptible to the action of the bacterial toxins.

* Presented at the meeting of the American Association of Pathologists and Bacteriologists, Cleveland, Ohio, April 2, 1931.

Birger and Rawitsch-Birger (7) noted that the action of the bacterial toxins on tissues of isolated organs was similar to that of other poisons but that there was a definite delay in its development. The action of diphtheria toxin was less marked at room than at body temperature. Between 19° and 22°C. the spasms induced by tetanus toxin ceased. Friedheim (8) studied the effect of *B. anthracis*, and Tannenbergs (9) that of the pyogenic cocci.

Krontowski (10) and his collaborators (11) have contributed further important observations on the deleterious action of diphtheria toxin on the growth and metabolism of the cells in tissue culture.

All this experimentation has been directed to the effect of the toxin on the tissues—the dosage that influenced growth or inhibited metabolism as determined by the consumption of sugar and liberation of lactic acid—together with the neutralizing action of antitoxin under various conditions. Also, the effect on the tissues of different animal species susceptible and insusceptible to the bacterial toxins has been tested in these studies. The experiments have not as yet definitely revealed an adaptive response in the cells of tissue culture to the specific toxins, comparable to that obtained by immunization of the animal. Fischer (12), however, records observations indicating an adaptive action, or tolerance to the presence of a foreign protein, in cultures of chicken fibroblasts.

The changes in the toxin have not been studied nor have the conditions under which the toxin is altered been determined. This study extends our early investigations to include experiments to determine the effect on diphtheria toxin of fetal and adult tissue and of the living, growing tissue culture.

Experiments with Fetal and Adult Cardiac Tissue

The myocardial lesions of diphtheria, which have recently been described by Warthin (13) in considerable detail, suggested the selection of cardiac tissue, and the guinea pig was chosen on account of its well known susceptibility.

A preliminary experiment was done in which 2 M.L.D. of standard diphtheria toxin were exposed to an emulsion of fresh embryonic cardiac tissue in Locke's solution, for neutralization.

The tissue was thrown down by centrifugalization. One 250 gm. guinea pig was inoculated with the top half and a second, of like weight, with the lower half of the contents of the centrifuge tube. The time required to produce death indi-

TABLE I
Effect of Exposure with Embryonic and Mature Cardiac Tissue upon the Potency of Diphtheria Toxin

Tissue emulsion (0.5 cc. used)	Diphtheria toxin dilution	Exposure for neutralization	Preparation of inocula	Method of inoculation	Results
0.1 gm. of embryonic guinea pig cardiac tissue in 0.5 cc. Locke's solution	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solution	3 hrs. at 37.5°C. in the dark	Centrifugalization, 3 min. at low speed. Supernatant fluid drawn into syringe with hypodermic needle. Sediment washed in 0.5 cc. Locke's solution, centrifugalized, and supernatant fluid drawn into same syringe Sediment washed in 0.1 cc. Locke's solution, centrifugalized, supernatant fluid drawn into unriused syringe Sediment suspended in 1 cc. Locke's solution	Subcutaneous Intracutaneous Subcutaneous	Death in 91 hrs. *No skin reaction No reaction
0.1 gm. of mature guinea pig cardiac tissue in 0.5 cc. Locke's solution	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solution	4 hrs. at 37.5°C. in the dark	Centrifugalization 3 min. at low speed. Supernatant fluid drawn into syringe with hypodermic needle. Sediment washed in 0.5 cc. Locke's solution, centrifugalized, and supernatant fluid drawn into same syringe Sediment washed in 0.1 cc. Locke's solution, centrifugalized, supernatant fluid drawn into unriused syringe	Subcutaneous Intracutaneous	Death in 98 hrs. *No skin reaction
Control	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solution		Syringe rinsed in 0.5 cc. Locke's solution	Subcutaneous	Death in 91 hrs.

White guinea pigs, 250 to 255 gm., were used.
* A typical Schick reaction results from 1/500 M.L.D.

cated that the first guinea pig had received considerably more, and the second guinea pig considerably less than 1 M.L.D., as would have been the case if the toxin had remained in the liquid, and the tissue in the bottom of the tube had merely replaced a volume of toxin.

Later tests were devised to give more clear-cut results; mature guinea pig cardiac tissue was included for comparison with the embryonic cardiac tissue, since the amount of actual muscle tissue in the embryonic hearts was unknown. The accompanying Table I records those tests. The potency of diphtheria toxin which had been in contact with such tissue was in no way lessened. Cardiac tissue of guinea pigs had no neutralizing or binding action for diphtheria toxin. In contrast, as appears from the following experiments, the growing cells in tissue cultures possess the power of completely neutralizing, binding, or destroying the diphtheria toxin so that the tissue of normal guinea pigs is wholly protected from the usual effect of the toxin.

Experiments with Embryonic Cardiac Tissue Cultures

Tissue cultures of embryonic guinea pig cardiac muscle were grown *in vitro* according to the method described by Hoppe (14), except that instead of one fragment of tissue being cultured, eight were placed in each slide equidistantly. The area to be covered with growth was about 113 sq. mm. Great care was taken to have the pieces of tissue adhere to the surface of the clotting medium but not to be submerged in it, since it was desirable that the growth should spread over the surface.

When the surface had become almost covered with tissue, which required 2 or 3 days, it was washed with embryo extract which was immediately removed with a capillary pipette, and then the cultures were exposed to 0.1 cc. of a solution of diphtheria toxin so diluted with Locke's solution that the 0.1 cc. contained exactly 1/500 M.L.D. of toxin.

Control preparations of clotted medium were also covered with like doses of toxin solution. Other controls, with and without tissue cultures, were prepared, to which the toxin solution was added after its potency had been destroyed by heating to 100°C. for 3 minutes and cooling. The cultures and the control preparations were all returned to the incubator and left for 48 hours.

White guinea pigs weighing from 250 to 275 gm. were carefully shaved on the ventral surfaces. The toxin solution was completely withdrawn from the growing tissue cultures and the control preparations into 0.5 cc. syringes graduated to tenths of a cubic centimeter and fitted with hypodermic needles, gauge 26. A separate syringe was provided for each preparation. These 0.1 cc. doses were injected intracutaneously into the guinea pigs. The tissue cultures were returned

to the incubator where they continued growing and pulsating. The guinea pigs were kept under close observation.

Guinea pigs injected with the toxin solution which had been exposed to growing tissue preparations showed no skin reaction. Guinea pigs injected with the toxin solution from control preparations, but without the presence of growing tissue, showed typical skin reactions. Guinea pigs injected with the toxin solution which had had its potency de-

TABLE II

*Effect of Exposure with Growing Tissue upon the Potency of Diphtheria Toxin.
Protocol of a Typical Test*

Amount	Toxin		Skin reaction
	Dose in Locke's solution	Exposed for 48 hrs. at 37.5°C.	
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture medium without tissue present	Reaction like Schick test
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture growing on culture medium	No reaction
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture growing on culture medium	No reaction
1/500 M.L.D. standard diphtheria toxin	0.1	Heated 100°C. for 3 min. and cooled. On tissue culture medium without tissue present	No reaction

For this test a normal white guinea pig, 250 gm., was used.

Four inoculations were made: anterior right, anterior left, posterior right, posterior left.

stroyed by heat showed no skin reaction. One guinea pig was used for several intracutaneous injections. Table II is a protocol of a typical test.

The tissue cultures were uninjured by the presence of the diluted toxin, and if they were occasionally washed with embryo extract after the removal of the diluted toxin, they could be kept living and used for repeating the experiment after several days. On the other hand,

the diluted diphtheria toxin required most careful handling at all times in order to avoid deterioration.

SUMMARY AND CONCLUSIONS

As determined by the intracutaneous test in guinea pigs, diphtheria toxin is not altered in the presence of cardiac tissue obtained from the fetal or from the adult heart of the guinea pig.

Tissue cultures were apparently uninjured by the presence of the toxin in the dilutions used in these experiments, and, when washed with embryo extract after removal of the diluted toxin, continued to grow.

Embryonic guinea pig cardiac muscle tissue growing in cultures *in vitro* possesses the power of neutralizing, binding, or destroying diphtheria toxin so that it is no longer toxic for normal guinea pigs.

Such neutralization takes place through the intervention of growing tissue and is a property which is lacking in similar surviving tissue not in a state of cultivation.

Thus, it appears that the living, growing cells of the tissues neutralize or destroy limited quantities of toxin; only when the quantity of toxin exceeds a certain limit is its action injurious.

REFERENCES

1. Wadsworth, A. B., and Vories, R., *J. Immunol.*, 1921, **6**, 413.
2. Wassermann, A., and Takaki, T., *Berl. klin. Woch.*, 1898, **35**, 5.
3. Levaditi, C., *Arch. Zellforsch.*, 1928, **6**, 187.
4. Burrows, M. T., and Suzuki, Y., *J. Immunol.*, 1919, **4**, 1.
5. Mendeléeff, P., *Compt. rend. Soc. biol.*, 1929, **102**, 949.
6. Kimura, R., and Ishii, T., *Z. Immunitätsforsch.*, 1930, **67**, 111.
7. Birger, O., and Rawitsch-Birger, H., *Z. Immunitätsforsch.*, 1926, **46**, 183.
8. Friedheim, E. A. H., *Compt. rend. Soc. biol.*, 1928, **99**, 1467.
9. Tannenberg, J., *Arch. Zellforsch.*, 1930, **9**, 258.
10. Krontowski, A. A., *Centr. Bakt., I. Abt., Orig.*, 1930, **117**, 328.
11. Krontowski, A., and Jazimirska-Krontowska, M., *Compt. rend. Soc. biol.*, 1929, **102**, 293.
12. Fischer, A., *J. Exp. Med.*, 1922, **35**, 661.
13. Warthin, A. S., *J. Infect. Dis.*, 1924, **35**, 32.
14. Hoppe, E. N., *Arch. Zellforsch.*, 1928, **7**, 352.