# THE ACTION OF TOXINS FROM PATHOGENIC ANAEROBES ON TISSUE CULTURES*

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With the development and refinement of methods for the cultivation of isolated animal tissues, numerous investigators became interested in studying certain questions in infectious pathology, using the "infectious pathogen–animal cell" model in vitro. Essentially, these investigations were devoted to studies of viral infections. At the same time, a small number of works are encountered in the literature dealing with the action of bacterial exotoxins on tissue cultures: mainly diphtherial; in individual cases, staphyllococcal and the pathogens of anaerobic wound infections [1, 3-6].

The purpose of this investigation was to study the cytotoxic action of toxins from various pathogenic anaerobes in tissue cultures. The following anaerobes were used: Cl. tetani, Cl. botulinum, Cl. perfringens, Cl. oedematiens, Cl. septicum and Cl. histolyticum.

## METHODS

Six types of tissue cultures were used in the experiments: cell strains HeLa, HLS, Detroit-6, monkey heart fibroblasts (SOTs), muscle tissue from human embryo, and preliminarily trypsinized tissue from 11-day old chick embryos.**

Cultures of the cells were first grown for 3–5 days in test tubes at 37°C, using the nutrient medium No. 199 supplemented with 20% beef serum, or Hanks' solution supplemented with 20% horse serum (HeLa strain). The initially trypsinized tissue from the chick embryos was cultivated for 24 hours in Hanks' solution containing 7.5% beef serum (the N. N. Dobronravova modification).

The toxins from the pathogenic anaerobes indicated above were diluted with an 0.8% solution of sodium chloride, or 2% beef serum in Hanks' solution. Then, 0.2 ml of the diluted material was introduced into the test tubes with the tissue cultures, containing 1.8 ml of the nutrient medium. Simultaneously, we added antibiotics–penicillin and streptomycin–using 5 units of each per ml of medium. In preliminary experiments, it was established that the antibiotics do not manifest a noticeable effect on the tissue cultures in these doses, while they do, however, inhibit reproduction of the microbes during the period necessary for evaluation of the experimental results.

Once the toxins were added, the test tubes containing the tissue cultures, along with the controls (not containing toxin), were again placed in an incubator at 37°C. Observation of the cultures was carried out over the course of 5–7 days, with daily inspection using low magnification (10 × 7).

## RESULTS

Through these investigations, it was established that tetanus toxin and botulin toxin types A, B, C and E do not show any visible cytotoxic effect on the 6 indicated types of tissue cultures, even when used in large doses. These observations correspond to the data in the literature [1], and confirm the concept of these toxins as specifically "neural" poisons.

* Presented with a film demonstration at a meeting of the Moscow Division of the All-Union Society of Microbiologists, Epidemiologists and Infectious Disease Experts on February 23, 1961, and at the Scientific Conference of the I. I. Mechnikov Moscow Institute of Vaccines and Sera on March 14, 1961.

** Tissue cultures for our experiments were prepared by N. N. Dobronravova and M. I. Klimanova.

In contradistinction to the tetanus and botulin toxins, those from the group of pathogens associated with anaerobic wound infection—Cl. perfringens, Cl. oedematiens, Cl. septicum and Cl. histolyticum—show a clearly manifested cytotoxic action on the cultures of initially trypsinized tissue from chick embryos and on the cultures of epithelial cells of the HeLa strain.

The results of the experiments are presented in the table.

The Action of the Toxins on the Different Tissue Cultures (according to the results of 3–5 trials)

| Type of toxin | Dose for mice (in Dcl) | Type of tissue culture | | | | | |
|---|---|---|---|---|---|---|---|
| | | Chick embryo tissue | HeLa strain | HLS strain | Detroit-6 strain | SOTs | Human embryo muscle tissue |
| Cl. perfringens | 1 | + | + | — | — | — | — |
| Cl. oedematiens | 1 | + | + | — | — | — | — |
| Cl. septicum | 1 | + | + | | | | |
| Cl. histolyticum | 1 | + | + | | | | |
| Cl. tetani | 1 000 | — | — | — | — | — | — |
| Cl. botulinum A | 1 000 | — | — | — | — | — | — |
| » » B | 200 | — | — | — | — | — | — |
| » » C | 2 000 | — | — | — | — | — | — |
| » » E | 200 | — | — | — | — | — | — |

Note. +)presence of a cytotoxic effect; —)absence of a cytotoxic effect.

Upon inspection of the test tubes through the microscope, the cytotoxic action of the indicated toxins on the chick embryo tissue culture was reflected by obvious destruction of a large number of cells. A portion of the cells were not destroyed, but they appeared more sharply contoured than the controls; the cell layer covering the wall of the test tube was not continuous, and in certain cases, it peeled away from the side. These changes in the tissue cultures were already clearly visible 3 hours after contact with the toxin.

Specificity of the cytotoxic effect was confirmed by the ability to prevent its occurrence through the introduction of the corresponding antitoxin, or the preliminary heating of the toxins for 15 minutes at 100°C.

The toxins apparently do not injure the cells instantly, since neutralization of the toxin with serum antitoxin any time within the first 15–30 minutes after its introduction into the tissue culture protects the latter from disruption and death. The cytotoxic effect is also avoided by removal of the toxin from the cultures through their being washed with nutrient medium during the first 15–30 minutes. Neutralization of the toxin, or washing it out, at a later period after contact with the tissue no longer can prevent the cytotoxic effect.

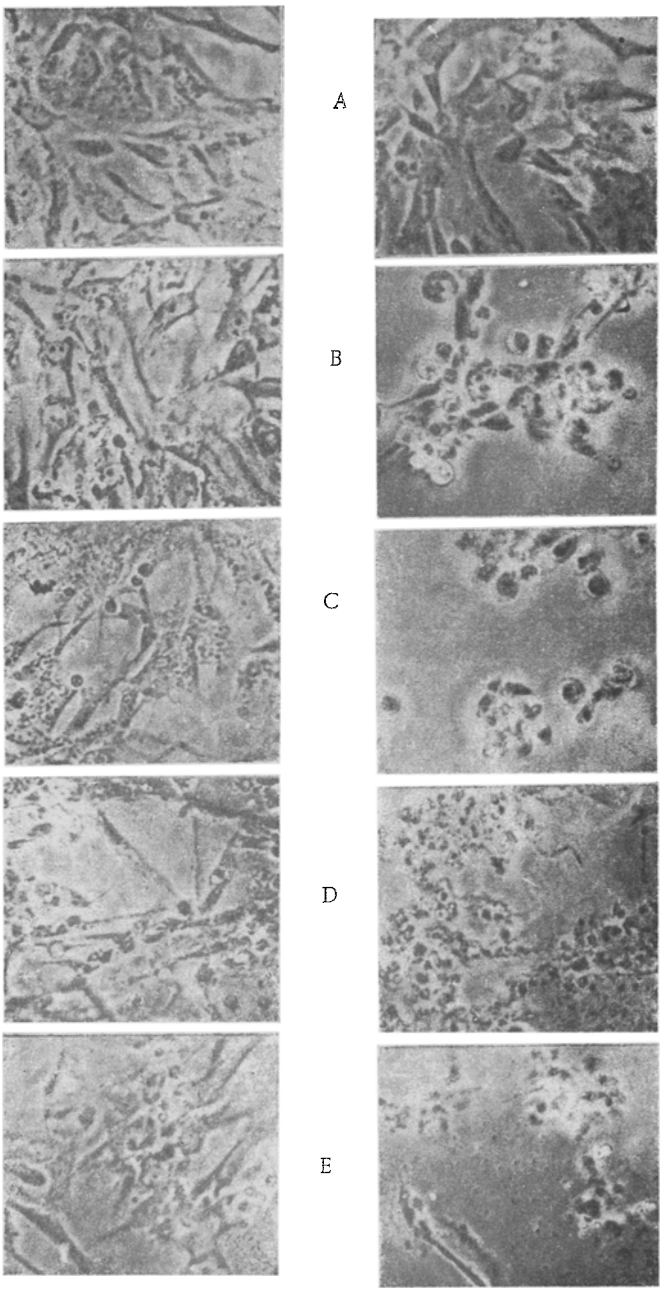
For a more detailed and deeper study of the changes in the tissue cultures caused by the toxins we used the method of time-lapse cinematography.

In this case, the cultures of initially trypsinized chick embryo tissue were grown in a special chamber, adapted for in vitro observations of the cells.

The pictures were taken with the aid of two microcinematographic cameras, which permitted simultaneous recording of changes in the tissue culture exposed to the toxin and the culture without the toxin.

The photographs were taken with a magnification of 40 × 10, at a frequency of one frame per 8 seconds, which permitted us to reproduce the process on the screen at a rate accelerated by a factor of 200. Individual frames obtained in these experiments are presented in the figure.

The figure clearly shows the cell changes in the tissue cultures arising from exposure to the different toxins over the course of 3 hours. However, according to statistical analysis, as well as judging from individual microscopic observations, we cannot demonstrate a significant difference in the action of the 4 trial toxins. A completely different picture is observed on the screen, using the cinematographic investigation: the dynamics of the tissue culture changes, and the death of their cells as a result of the different toxins is far from monotypic.




Cytotoxic effect of the toxins from the pathogens of anaerobic wound infections on an initially trypsinized chick embryo tissue culture. Frames from a film; phase contrast microscope; magnification 40 × 10. I) action of the toxin in the course of 1 hour; II) action of the toxin in the course of 3 hours; A) control; B) Cl. perfringens; C) Cl. oedematiens; D) Cl. septicum; E) Cl. histolyticum.

In the most general outline, the process observed on the screen, accelerated 200 times, appears in the following fashion:

Under the influence of the toxin from Cl. perfringens, the intercellular connections in the culture are destroyed, the cells of the tissue become rounded, their contours become more sharply defined, movement of the cytoplasm gradually ceases, a portion of the cells is destroyed, and another portion accumulates into formless conglomerations (see figure, B).

Under the influence of the toxin from Cl. oedematiens, destruction of the cell layer also occurs, along with disruption of the intercellular connections, but in this case an intense movement of the cytoplasm of the cells is highly characteristic, its "boiling", preceding the death of the cell. The cells "agonize violently"; the final picture of their death is similar to the previous description (see figure, C).

The death of the tissue under the influence of the toxin from Cl. septicum is also unique. The initial process of disruption of the intercellular connections rapidly ceases; the tissue layer, and the cells composing it, become motionless, as though fixed in a state prior to the onset of the marked destructive changes. Following this, the cells shrivel and lose their normal configuration; in the final stage, accumulations of these dying cells form so-called cellular detritus (see figure, D).

The toxin of Cl. histolyticum is similar to the toxin of Cl. perfringens in its action on the tissue culture, but it causes a more intense destruction of cells (see figure, E).

The investigations performed showed that tetanus and botulin toxins do not manifest a cytotoxic action on the 6 different types of tissue cultures tested. This confirms the obtaining concept that the "site of action" of these toxins is the nervous system. The toxins of the 4 species of anaerobic wound infection pathogens are characterized by vividly manifested cytotoxic actions against certain types of tissue cultures. In this case, we were unable to relate the cytotoxic action to the character of the tissue: the toxin of Cl. perfringens, for example, disrupts chick embryo tissue cultures, consisting, basically, of mesenchymal elements, and likewise affects the HeLa strain epithelial cultures, but does not act on the culture of human embryo muscle tissue or SOTs, nor on the HLS and Detroit-6 strains of epithelial cultures.

The use of the method of time-lapse microcinematography makes it possible to demonstrate dynamically the specific characteristics of the action of 4 anaerobic wound infection pathogens on a tissue culture.

## SUMMARY

As shown, tetanus and botulin toxins had no cytotoxic effect on the 6 tested types of tissue cultures. Cl. perfringens, Cl. oedematiens, Cl. histolyticum and Cl. septicum toxins produced a marked cytotoxic effect on the cultures of the HeLa strain and on the primarily trypsinized tissues of chick embryo. The use of the method of slow microcinephotography has demonstrated the specific features of the toxin action (in dynamics) of 4 causative agents of the wound anaerobic infection on tissue culture.

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