A COMPARATIVE STUDY OF VACCINE AND VIRULENT ANTHRAX STRAINS IN A TISSUE CULTURE OF A HUMAN EMBRYO

TRANSLATION NO. 1045

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

A COMPARATIVE STUDY OF VACCINE AND VIRULENT ANTHRAX STRAINS IN A TISSUE CULTURE OF A HUMAN EMBRYO

[Following is a translation of an article by N. N. Ginsburg and Yu. M. Fedotova, Gamaleya Institute, AMN, USSR, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology, and Immunobiology), No. 11, 1963, page 3-6.It was submitted on 14 August 1962. Translation performed by Sp/6 Charles T. Ostertag, Jr.]

The study of a number of problems of infectious pathology and immunity in anthrax by using in vitro tissue cultures began in the early twenties, shortly after this method was proposed. At that time, the tissue cultures which the investigations used were explants cultivated in vitro. An evaluation of the results of experiments in which such a type of tissue culture was used represented significant difficulties due to the structural complexity of the explants with numerous cellular elements entering into them and the difficult to analyze interrelations emerging between the explant on the whole and the bacteria under study.

With the appearance of the method of single layer cultures or as it is acceptedly called today the cell culture method (Suim, 1959), it became possible to study the above mentioned problems on a cellular level which is simpler from the point of view of experimental analysis. Nevertheless, in spite of this, much from what was done in the works of the twenties even at the present time didn't lose a certain importance since it appeared as the first exploration in a little investigated area of studying the problems of infectious pathology and immunity in tissues in vitro.

In connection with the problem of natural nonsusceptibility, Friedheim (1928) studied the in vitro reaction of tissues from animals which were susceptible (mice) and nonsusceptible (chickens) to anthrax infection. He used tissue cultures (explants) of mice and chick embryos and "stable"

subcultured tissue cultures of these animals. The author observed a complete indifference of the explants to the presence of the anthrax causative agents in the test tube. He didn't notice any differences in the reaction of explants from tissues of animals susceptible and nonsusceptible to anthrax.

Singer and Hoder (1929) studied the influence of extracts from anthrax bacilli, of extracts from the spleen of an animal infected with anthrax, and from anthrax edematous fluid on the activity of leucocytes and lymphocytes of a spleen explant and on the intensity of its peripheral growth. The authors discovered that the extract from anthrax bacilli not only didn't exert a negative influence on the activity of leucocytes and lymphocytes, but even stimulated the growth of the explant. An extract from the spleen of a guinea pig infected with anthrax also exerted a stimulating influence on the growth of the explant, but at the same time it inhibited the exit from the explant of polymorphonuclear leucocytes (without influencing the exit of lymphocytes). Anthrax edematous fluid inhibited the growth of explants and impeded the exit of leucocytes and lymphocytes. The authors expressed the assumption that the products formed in the organism during infection caused a selective affection of elements from the reticuloendothelial system.

In connection with the problem of immunity in anthrax, Gakh and Boroday (citation from Krontovskiy) studied the reaction of skin explants from immune and nonimmune rabbits to anthrax infection. They weren't able to observe any existing difference in the reaction of the explants and in the behavior of the anthrax bacilli. The bacilli germinated the skin explants of both the immune and normal animals to a similar degree.

The present work is devoted to a comparative study of the in vitro interrelations between the cells of tissue cultures (cells) of a human embryo and anthrax bacilli with a various degree of virulence.

It is known that the virulence of anthrax bacilli is determined by two factos -- the capsule and the toxin. In the work it was assumed to look into their role in the process of the direct interrelations (contact) of the bacilli with the cells in a test tube. For the specified aim a young culture of anthrax causative agent of different virulence was added to a cell culture of a human embryo which was cultivated on an artificial nutrient medium. Thus, a system was created where in a common medium of residence (close to optimal for both partners) the multiplying and metabolizing cells of the human embryo and the anthrax causative agents existed together for a known time and interacted directly (by direct contact) and through the medium of residence. In this we studied, on the one

hand, the nature of contact of the bacilli and the cells, particularly its morphological expression, and on the other hand, the cytopathic action of the cells, if such exists, and primarily their ability for phagocytosis or any other antibacterial action.

The cell cultures of a human embryo were cultivated on plates of cover glasses enclosed in test tubes. The nutrient medium was a solution of lactalbumin hydrolyzate with 10% inactivated calf serum and 0.002% phenol red; the pH of the medium was 7.4.

For infection with the anthrax causative agent, we mainly used tissue cultures of a 3-5 day growth, while a solid layer of cells still hadn't formed on the glass and thus the limits of the cells and their morphology could be seen clearly in the preparation.

In the work we used two anthrax strains -- the highly virulent capsule strain No 7, and the capsuleless STI-1 vaccine strain. In a series of preliminary tests the optimum dose was worked out for infecting the tissue cultures (2 million microbial cells in a test tube) which would permit observation of the system for 6-8 hours. This dose was used in the experiments both with vaccine and virulent strains.

After changing the medium and a preliminary washing the cell cultures were infected with young 20-24 hour cultures of anthrax bacilli which were incubated in Hottinger broth and diluted to the necessary concentration by the medium for incubating the cells. After infection, the test tubes were placed in an incubator (37°) for 6-8 hours. After a determined interval of time the plates with cell cultures were withdrawn from some of the test tubes and smears were made from the culture fluid, or if this wasn"t necessary seedings were made.

The smears and cell cultures on the plates were fixed with methyl alcohol and stained according to Romanovskiy - Giemsa. Before fixing, the cultures on the glasses were thoroughly washed with a physiological solution. This manipulation was necessary for determining the ability of the anthrax bacilli to become fixed in cell cultures and for clearing up the "stability" of this fixation. In the process of washing off, as a rule almost all of the bacilli are removed from the glass and the culture with the exception of those which are tightly affixed to the cells.

In these same periods in test and control test tubes the pH (based on phenol level) and turbidity (based on optical standard) were determined, that is indicators of the intensity of metabolic processes taking place in the system, and the accumulation of bacteria in the medium. A medium identical to the one being tested served as a control in the tests. It contained lactalbumin hydrolyzate with 10% inactivated serum and phenol red. The test tubes with the control medium were infected, incubated at 37° and processed similar to the test ones.

As a result of the work performed it can be considered established that interrelations of the bacteria and cells of the culture in vitro are formed out of interdirected processes. The influence of the first was expressed in their cytopathic effect on the culture cells, the influence of the second in the action on the bacteria by some apparently nonspecific bacteriolytic factors and in phagocytosis of the anthrax bacilli by specific elements of the cell cultures.

The enumerated interdirected processes in the system were considerably different based on expressiveness in the case of the vaccine and virulent strains.

The bacilli of the vaccine strain behaved completely indifferent to the cells, they weren't fixed on them, and multiplying (metabolizing) in direct proximity didn't cause expressed degenerative changes in them. This went on until in the medium for the common residence of vaccine bacilli with the cells no changes take place (for example, sharp disturbance of the pH, accumulation of exchange products, impoverishment of the medium, etc.) which entail the death of all cells with their desquamation from the glass as if from secondary causes. With the initial concentration of microbes equal to two million, these effects begin in a period in excess of 7-8 hours. Until this time the bacilli of the vaccine strain intensively multiplied in the liquid phase of the cell culture. By six hours of combined cultivation their concentration in the test tubes reached >10 units of turbidity based on the optical standard of the Government Control Institute which in microbial cells conforms to $\simeq 1 \times 10^8$ bacilli of anthrax in 1 ml of culture fluid. By this time the pH of the medium had dropped to 6.0. However, in spite of such a large amount of bacteria in the system the morphology of the cells didn't change and there were no bases for the conclusion that the cells underwent any harmful specific influence.

In contrast to this the virulent anthrax bacilli exerted a sharp cytopathic effect on the cells. They were fixed on the cell cultures (fig. 1), multiplied, and covered them with a thick network of little chains solidly attached to them. As a result, degenerative changes appeared in the cells. Their cytoplasm was vacuolized and took on a foamy design. Communication between cells was ruptured. The prolongations drew up and the cells crumpled up. Pyknosis of the nuclei was observed (fig. 2). By the end of the observation (6-8 hours) the cells perished and, covered with anthrax bacilli, desquamated from the glass.

Thus, in experiments with virulent strains a distinct fixation of capsular anthrax bacilli was noted on the tissue cells with an expressed toxic effect of the bacilli on the cells. These facts permit a new look at the role of the capsule as a factor of virulence for anthrax bacilli.

Apparently, its role doesn't amount to just the function of protecting the bacilli from phagocytosis and bactericidal substances from the tissues of an organisms as this is considered up until now. Thanks to it, virulent anthrax bacilli turned out to be capable of fixation on cells and thus come into close contact with the latter, actively affecting them with a second factor of virulence - the toxic factor. Vaccine cells devoid of a capsule are also devoid of the possibility of affecting the tissue cells.

As was already said the cells in their turn also exert an influence on the bacilli. We studied the possibility of phagocytosis of the anthrax bacteria by various elements of a cell culture from a human embryo. In the culture, cells of the fibroblast type predominated absolutely, but a certain amount of epithelial type cells are encountered as well as monocytes and lymphocytes.

In our experiments we didn't observe phagocytosis of anthrax bacilli either by cells of the fibroblast type or epithelium like cells, though in literature at the present time there are facts about their capability for phagocytosis of other species of bacteria. Only cells of the monocyte type possessed the capability to phagocytize (fig. 3 and 4). It is noteworthy that these actively phagocytizing cells are obtained in vitro cultures from 6-12 week human embryos.

It is necessary to add that we were able to observe phagocytosis clearly only in cultures with the vaccine anthrax strain. The exposure of phagocytosis of virulent bacilli was difficult due to the fixation of the bacilli on the cells.

Along with phagocytosis, in a great number of experiments it was possible to observe a partial disintegration of bacilli of both the vaccine and the virulent strain which were located on the cells of the culture and in direct proximity to them.

The nature of the damage to the bacilli bore a various morphological expression. Some of these became swollen, their tinctorial properties were reduced and then they were lysed (fig. 6). On the flat side of others circular protrusions emerged, apparently as a result of the flowing out of part of the cytoplasm of the bacteria through the damaged membrane (fig. 5). Some nonspecific bacteriolytic factors caused the bacterial lysis which was observed. These factors were either liberated by the cells of the tissue culture into the medium or arrived there as a result of cell disintegration. However that may be, it must be noted that the cells were not indifferent by far in their behavior to contact with the bacilli and responded to it with a total of apparently protective reactions.

In analyzing the differences in the intensity of multiplication of virulent and vaccine strains of anthrax bacilli in test tubes with cell cultures and in controls it was demonstrated that with the remaining conditions equal the vaccine bacilli multiplied in the liquid phase of a cell culture more intensively than the virulent ones. Besides this the bacilli of the vaccine strain multiplied twice as intensively in the liquid phase of the cell culture than in a control medium.

CONCLUSIONS

1. Distinct differences were obtained in the behavior of virulent and vaccine strains of anthrax bacilli in cell cultures of a human embryo. In experiments with virulent bacilli there was a clear determination of the action of both factors of virulence -- the capsule and toxin.

2. Data was obtained concerning the presence in the cells of a human embryo in vitro culture of mechanisms of natural antibacterial protection.

LITERATURE

1. Krontovskiy, A. A., Tsentr. Med. J., 1929, v.4, page 217.

2. Friedheim, E. A. H. C. R. Soc. Biol. 1928 v.99, pages 1299, 1467.

3. Singer, E., Hoder, F., Z. Immun. - Forsch., 1929, Bd. 60, page 429.

4. Swim, H. E., Ann. Rev. Microbiol., 1959, v. 13, page 141.

5. Thal, E., Untersuchungen uber Pasteurella pseudotuberculosis, Lund., 1954; Nord Vet. - Med., 1954, v. 6, page 829.

PHOTO CAPTIONS

Fig. 1. Fixation of bacilli of virulent anthrax strain No. 7 on the cells of an in vitro tissue culture (6 x 8).

Fig. 2. Degeneration changes in the cells of a tissue culture in the presence of anthrax bacilli of virulent strain No. 7 (6 x 90).

Fig. 3.&
4. Phagocytosis of anthrax bacilli of the STI - 1 vaccine strain in a cell culture of human embryo in vitro (6 x 90).

Fig. 5. The most typical form of disintegration of anthrax bacilli in a cell culture of human embryo in vitro (6 x 90).

Fig. 6. One of the forms of lysis of anthrax bacilli in a tissue culture of the human embryo in vitro (6 x 90).

[The following English summary appears with the Russian article.]

A study was made of the results of interaction (interdirected processes and interconditioned reactions) between the cells of the human embryo in vitro cultures and virulent and vaccine anthrax strains. Distinct differences were revealed in the behavior of virulent and vaccine bacilli in the cellular culture. In experiments with virulent anthrax bacilli a distinct action of both virulent factors was determined -- of capsule, of the toxin. At the same time data were obtained on the presence of the natural antibacterial protection mechanisms in vitro culture of human embryo cells. ( )