THE SERUM NEUTRALIZATION OF HEMOTOXINS

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Early in the history of bacteriology attention was directed toward certain very labile bacterial toxic substances, produced to a variable extent by many organisms, which cause hemolysis of blood. Without attempting to review the work dealing directly or indirectly with such hemotoxins, it appears one may safely state that no very satisfactory conclusion has been arrived at as to what part if any these toxic materials play either in the natural disease and artificial immunization on the one hand, or in the production of the best antiserums on the other hand. In the production of commercial bacterial vaccines, a quite general attempt is made, as has been done in the past, to maintain stock cultures of some of the more highly invasive bacteria in a state of high virulence for animal tissues and to maintain hemolytic properties in some instances. Test tube neutralization of bacterial hemotoxin has been used, together with mouse protection, agglutination, et cetera, to evaluate potency of antiserums such as those produced with hemolytic Streptococci.

As long as the hemolysis of erythrocytes appeared to be the only outstanding characteristic of such toxic substances, their importance in infectious disease may have been underestimated. However, some hemotoxins such as certain highly potent ones produced by *Staphylococcus aureus* have been found to be capable of causing degeneration of leucocytes, and of bringing about massive necrosis of fixed tissues and death in experimental animals. These latter facts together with changes in opinion concerning the best antibody content of antiserums other than the classical antitoxins have to some extent possiblly reopened the question of the importance of bacterial hemotoxins and other very labile antigens in immunity. The Bundaberg disaster in which several human fatalities resulted from the injection of living Staphylococci which were later found to produce a great amount of hemotoxin also called attention to a possibly wider role which the hemotoxins may play in pathology than hitherto suspected.

In this paper results of tests for the examination of hemotoxins from some human pathogens are given. The neutralization of these hemotoxins by antiserums is described, and some comments are made upon this field of immunology.

Experimental. Hemotoxins were produced from Streptococcus, Staphylococcus, and Tetanus cultures. The organisms were either grown for a few hours in high grade veal infusion broth or for a longer time, one to five or six days, in similar broth suitably buffered. Saline washings from agar growth also yield satisfactory hemotoxin. These cultures, on removal from the incubator, were cleared with the centrifuge and then passed through a Berkefeld N filter.

The potency of these hemotoxins was estimated as rapidly as possible as follows. One-half cubic centimeter of each of a series of dilutions of hemotoxin was mixed in tubes with one-half cubic centimeter of five percent suspension of washed rabbit erythrocytes. These mixtures were placed at 37°C. for an hour and readings made in terms of degrees of hemolysis. The hemolytic titers of all of our filtrates, including about twenty-four test lots, varied from fifty to two hundred hemolytic doses or units per cubic centimeter. In general, the Staphylococcu

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filtrates were strongest, with Tetanus filtrates intermediate and Streptococcus filtrates weakest.

Our experience in immunization of both small laboratory animals and also horses for curative serum shows that fresh active hemotoxin is highly antigenic, and high titered serums are rather readily produced. There is evidence also that after the hemotoxin has lost its activity spontaneously it still immunizes well. This is in harmony with the fact that spontaneously inactivated hemotoxin can be made highly hemolytic again through the use of reducing substances, and indicates that in the inactive state perhaps it acts much like a sort of toxoid. For all of our test tube neutralization of hemotoxin with antiserum, however, we have regularly used freshly produced hemotoxin. During the time each separate lot of this was being tested, the bulk of the lot was kept in the icebox. As soon as the unitage was determined by the tests described above, sufficient hemotoxin for neutralization tests was then diluted so that one-fourth cubic centimeter contained two hemolytic units. For the neutralization tests one-fourth cubic centimeter of diluted hemotoxin containing two units was run into each of a series of small test tubes and then one-fourth cubic centimeter doses of each of a series of dilutions of antiserum were added. These tubes, including proper controls, were incubated at 37°C. for one hour. Then one-half cubic centimeter doses of five percent suspension of washed rabbit erythrocytes were added to the tubes. Incubation was then carried out for an additional hour at 37°C. Readings were then made in terms of degrees of hemolysis, and therefore no hemolysis means complete neutralization of hemotoxin with antiserum. Various antiserums were found to contain from 4,000 to 16,000 units of antihemotoxin per cubic centimeter, the unit being that amount just neutralizing a hemotoxin unit.

It was found from these homologous and heterologous neutralization tests that there is a large amount of overlapping of reaction; for example, Tetanus and Staphylococcus antiserums neutralize Streptococcus hemotoxin. In other words, any one of the three antiserums (Streptococcus, Staphylococcus or Tetanus) will neutralize any one of the hemotoxins, but not always to the same extent. It appeared that the Streptococcus hemotoxins were most readily neutralizable by heterologous antisera, while Staphylococcus and Tetanus hemotoxins were less readily neutralizable in this way. Diphtheria antiserum was used throughout this work as a control since it has little if any antihemotoxic property. Antihemotoxic properties of raw serums are readily concentrated four or five times by the regular salting out methods.

The relation which these positive cross neutralization results may have to the use of antiserum curatively is obscure. There is no information as yet, for example, on the treatment of hemolytic Streptococcus infection in man with either Tetanus or Staphylococcus antiserum. Under ordinary conditions of production Tetanus antiserum appears to have a very constant high titer of antihemotoxin, and probably where special precautions are not taken in the preparation of filtrates the Tetanus filtrate will be found superior in hemotoxin content. This may be due in part to the protection from oxidation afforded the hemotoxin in this instance by the anaerobic cultivation.

We have observed that Streptococcus antiserum containing antihemotoxin can be specifically used with very good results in hemolytic Streptococcus bronchopneumonia in man while Pneumococcus antiserum without antihemotoxin has no effect. Of course the Streptococcus antiserum also contains other Streptococcus antibodies such as protective antibody, opsonin, et cetera, not present in the pneumococcus serum. However, the striking drop in temperature and sense of

well-being in Streptococcus pneumonias following the use of Streptoccocus antiserum may at least in part be due to the antihemotoxins. Recently we have received reports that our Streptococcus antiserum has been used with very good results in the condition in man known as blackwater fever. This is directly or indirectly caused or provoked by malaria, and a powerful hemotoxin is in some way produced in the body either by the parasites themselves or perhaps by Streptococci which may chance to be present. The hemotoxin, whatever its source, appears to be readily neutralized by the Streptococcus antiserum.

Discussion. Whether the bacterial hemotoxins play a considerable part in human infectious disease is still an open question. These materials resemble the classical toxins in many ways including their degree of lability, and their property of inciting large amounts of antihemotoxin in suitably treated animals. In view of the criticism of the practice of using for active immunization stock bacterial vaccines produced from cultures removed from the original lesions by a long series of subcultures upon artificial media, and sterilizing these by heat, one may suggest the following modification of procedure. The vaccine may be more surely like the natural virus if it is propagated preferably in the presence of human blood for at least part of the time. Its hemolytic action on human blood or its ability to grow in the presence of human blood may be tested, and thereby many useless organisms may be separated out. In order to preserve the labile antigenic fractions such as hemotoxns, the cultures may be devitalized with suitable germicide instead of being killed by heating. It appears that hemotoxins so prepared, and even when inactivated spontaneously by oxidation, are still highly antigenic and act somewhat as a toxoid.

In view of the general failure to obtain results fully up to expectation in the therapeutic use of antiserums containing only antibacterial body, such as agglutinin, it may be desirable to include in such serums more or less antihemotoxin in case the corresponding bacteria may be hemolytic. This may be brought about by injecting horses with cultures containing the maximum of hemotoxin and which have been devitalized with suitable germicide instead of being heat-killed. In this way a maximum of antibacterial body will be included in the serum, as well as immune substances incited by the more lable antigens which a given culture may produce.

Our experience in animal immunization shows that antihemotoxins are readily incited after treatment with potent hemotoxin. The latter, even when it has lost its activity spontaneously through age, is still a good antigen. The fact that hemotoxin generally appears very early in the growth of a culture may indicate that antihemotoxin may conceivably come into action very early in an infectious disease. Since very little is known about any reactions taking place in the very earliest stages of infection, hemotoxin-antihemotoxin studies merit further study, as do those reactions involving all very lable antigenic fractions.

Conclusions

1. Potent hemotoxins can be quite regularly produced from Streptococci, Staphylococci, and Tetanus bacilli. Through suitable animal treatment high titered antihemotoxins are obtained.

2. There is quite a wide range in cross or heterologous neutralization of hemotoxin with antihemotoxin.

3. Hemotoxins and other labile antigens merit further study as a means of improvement of prophylactic vaccines and in turn of certain antiserums other than the classical antitoxins.