STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

V. Production of Antihemotoxin by Immunization with Oxidized Pneumococcus Hemotoxin.

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INTRODUCTION.

It is known that active or reduced pneumococcus hemotoxin is antigenic since immunization with it induces a so called "antihemotoxin" which neutralizes the lysin (1). There is a marked lessening in the hemolytic activity of oxidized pneumococcus extracts (2), and it has seemed important to determine whether a loss in antigenicity accompanies the inactivation of the hemolytic property. Our investigation of this question has consisted in a comparison of the immunity response to the injection of reduced and oxidized pneumococcus extracts.

EXPERIMENTAL.

Methods.

Substances Injected.—A series of rabbits were immunized by injection of the following substances: (1) "reduced" or active pneumococcus extract which was stored in sealed tubes and subjected to a minimum exposure to air before injection; (2) oxidized or inactive extract which was exposed to air for 6 hours at 37°C. and then stored at 5°C.; (3) "vaccine" or heat-killed suspensions of pneumococci.

The reduced extract contained the active hemotoxin in high concentration, 0.001 cc. producing hemolysis when added to 2.5 cc. of red blood cells. The oxidized extract contained inactive hemotoxin and before each injection was always tested for the presence of traces of active lysin. That no significant amount of it persisted in the oxidized extract was proved by the fact that 0.3 cc. of the extract caused no detectable hemolysis when added to 2.5 cc. of blood cells. The results

in animals injected with the heat-killed bacterial suspension were included for purposes of comparative study. The bacterial suspensions were heated at 56°C. for 30 minutes. Young cultures were used and the suspensions were heated at once to minimize autolysis and cell solution.

Immunization.—The reduced and the oxidized sterile cell solutions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Four courses of injections were given, the doses gradually increasing from 0.1 cc. to a maximum of 2.0 cc., in the last series of injections.

The "vaccine" or heat-killed bacterial suspension was also injected intravenously at the same time as the sterile cell solutions. Four courses of six daily injections of 0.1 cc. of a concentrated suspension of the bacteria were given so that the animals received in all the equivalent of bacteria from 24 cc. of culture.

All of the rabbits were bled on the 10th day after the last injection.

Titration of Antihemotoxin in Immune Serum.—The method used in determining the antihemotoxin value of the immune serum consisted in adding various amounts of serum to a constant amount of hemotoxin and determining the amount of serum required to completely neutralize the lysin. The hemotoxin-serum mixtures were shaken and then incubated for 45 minutes to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated to determine the presence of free or unneutralized hemotoxin. The detailed procedure is given below:

Constant "Dose" of Hemotoxin.—The hemotoxin content of a sterile pneumococcus extract was determined by a preliminary titration. Three times the amount of extract found sufficient to completely hemolyze 2.5 cc. of a 1 per cent suspension of washed rabbit cells was chosen as the test "dose" of hemotoxin. The pneumococcus extract was then diluted to such a concentration that 1.5 cc. of the dilute solution contained this amount of lysin.

Hemotoxin-Serum Mixtures.—0.5 cc. of different dilutions of serum was added to the series of tubes containing 1.5 cc. of the dilute hemotoxin solution. Owing to the inhibitory effect on the lysin of certain constituents of normal serum, control tubes were included containing the sera of the animals from bleedings made before immunization. The mixtures of hemotoxin and serum were shaken and then incubated for 45 minutes at 37°C.

Final Hemolysis Tests.—At the end of the 45 minutes, the presence of free or unneutralized lysin was determined by adding 0.5 cc. of a 5 per cent suspension of rabbit cells to the hemotoxin-serum mixtures. These final hemolysis test mixtures were incubated for 1 hour and readings of hemolysis made after centrifugation.

Control of Lysin "Deterioration" during Incubation of Hemotoxin-Serum Mixtures.—It was necessary to rule out the possibility that the lysin might be inactivated by oxidation since, should this occur, it might be confused with the immunological neutralization. For this purpose, control mixtures of 0.5 cc. salt solution plus 1.5 cc. of dilute hemotoxin were incubated with the series. At the end of the incubation period, these mixtures were diluted one-half and one-third; and the usual amount of red blood cells was added after these dilutions of the

control lysin had been made up to the correct volume of 2.0 cc. The control test mixture containing one-half and one-third the amount of lysin used, always showed complete hemolysis which proves that none of the apparent lysin neutralization or inhibition can have been due to inactivation of hemotoxin by oxidation during the preliminary incubation period.

Absence of Visible Precipitation in the Incubated Hemotoxin-Serum Mixtures.—Titrations of immune serum for the presence of antibodies neutralizing hemotoxins or enzymes are often complicated by the fact that the tested sera contain precipitins for other proteins present in the cell solutions. If during the preliminary incubation of the serum and cell solution, a precipitation or flocculation occurs, it is always possible that the active substance may be carried down with the precipitated particles. Subsequent tests of mixtures in which this precipitation has occurred indicate an apparent inhibition or neutralization of the test substance although in fact the active substance may have been only mechanically removed.

No such source of error existed in our experiments; for there was never any visible clouding or precipitation. The point is important since the immune sera contain a precipitin for the pneumococcus protein present in the bacterial cell solution used to furnish the hemotoxin. When the serum and bacterial solution are less dilute than in mixtures made according to the above procedure, protein precipitation occurs.

Strength of the Antihemotoxin in the Serum of Animals Immunized with Oxidized (Inactive) and Reduced (Active) Pneumococcus Hemotoxin.

In the following experiment a comparison was made of the hemotoxin-neutralizing power of the sera of animals immunized with oxidized (inactive) hemotoxin and that of sera immunized with the reduced (active) hemotoxin. The serum of a rabbit immunized with a heat-killed suspension of intact pneumococci was included for comparison. The general procedure has been described under Methods. In this experiment, the immune sera were tested against hemotoxin derived from pneumococci of the same fixed type (Type II) as the cell solutions used for immunization. The results are given in Table I.

The marked neutralizing power of the sera of animals immunized with both the oxidized (inactive) and the reduced (active) solutions of pneumococci is evident in Table I. The production of an antihemotoxin by the injection of cell solutions containing the active hemotoxin has been shown in Cole's (1) studies. The new fact revealed in Table I is the production of a neutralizing antibody by immunization with oxidized, that is to say, inactive pneumococcus hemotoxin. It is

evident that the serum of the animals injected with an oxidized extract which contains no demonstrable active hemotoxin possesses approximately the same neutralizing action as does that of animals immunized with the reduced, or active, hemotoxin.

The animal immunized with the heat-killed suspension did not produce antihemotoxin. This is an interesting correlation with the similar failure of immunization with properly prepared "vaccines" to yield an antibody for pneumococcus protein which like the hemotoxin is an endocellular substance (4).

TABLE I.

Antihemotoxin in Sera of Animals Immunized with Reduced (Active) Hemotoxin and Oxidized (Inactive) Hemotoxin.

No. of rabbit	Immunized by injection with	Hemolysis by previously incubated mixtures of hemotoxin and serum						
		Immune serum					Normal serum, before immunization	
		Amount of serum					Amount of serum	
		0.01 cc.	0.006 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.05 cc.	0.01 cc.
1	Inactive hemotoxin in oxidized cell solution	0	0	0	0	0	++++	++++
2	" "	0	0	0	+	±	+++	++++
3	Active hemotoxin in reduced cell solution	0	0	0	0	±	+++	++++
4	" "	0	0	0	0	0	+++	++++
5	"Vaccine" (heat-killed suspension of intact cells)	+++	++++	++++	++++	++++	++++	++++

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

In the preceding experiment, the neutralizing capacity of the immune sera was tested against hemotoxin derived from the homologous type of pneumococci. Cole (1) has shown that the antihemotoxin produced by immunization with the active substance is not type-specific,

but is effective against hemotoxin derived from all types of pneumococci. It seemed of interest to determine if the antihemotoxin produced by immunization with inactive (oxidized) hemotoxin is likewise equally effective against the hemotoxin derived from heterologous types of pneumococci. The protocol of an experiment testing this question is presented in Table II.

As shown in Table II the antihemotoxin which is produced by immunization with oxidized or inactive Pneumococcus is only species-

TABLE II.

Species Specificity of the Antihemotoxin Produced by Immunization with Oxidized (Inactive) Pneumococcus Hemotoxin.

Serum of animal immunized against	Hemolysis by previously incubated mixtures of hemotoxin and serum					
	Hemotoxin from Type I Pneumococcus		Hemotoxin from Type II Pneumococcus		Hemotoxin from Type III Pneumococcus	
	Amount of serum		Amount of serum		Amount of serum	
	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.	0.01 cc.	0.002 cc.
Inactive hemotoxin in oxidized pneumococcus cell solution (Type II)	0	0	0	0	0	0
Active hemotoxin in reduced pneumococcus cell solutions (Type II)	0	0	0	0	0	0
"Vaccine" (heat-killed suspension of intact cells)	++++	++++	++++	++++	++++	++++

specific and not type-specific since it neutralizes the hemotoxin derived from the cells of heterologous types of pneumococci. A species-specific antibody is apparently the usual type involved in the neutralization of bacterial hemotoxins; for the hemotoxin from cholera vibrios which are type-specific by agglutination, likewise gives rise to a common neutralizing antibody (3). The same holds true of the neutralizing antibody for the true toxins formed by diphtheria, tetanus and Welch bacilli, although each of these bacterial species includes strains which exhibit type specificity in tests with agglutinating immune sera.

Comparison of the Inhibiting Effect of Normal and Immune Serum upon Digitonin Hemolysis.

The serum of normal animals possesses a certain inhibitory action upon pneumococcus hemotoxin. At least a part of this inhibition by normal or non-immune serum can be referred to lipoid constituents. In the case of tetanolysin, a number of investigations have proved that the increase in the neutralizing action of immune serum is not due to an increased concentration of the lysin-inhibiting constituents of normal serum, and it might be concluded by analogy that this is also true for the pneumococcus hemotoxin. However, in view of the fact that the antihemotoxin described in the present paper is produced by immunization with a non-hemolytic antigen, it seemed desirable to determine whether the increased neutralizing capacity is due to a non-specific substance which would inhibit the hemolytic activity of other lytic agents.

Digitonin was chosen as the lytic substance to test this question, largely because hemolytically active digitonin is known to combine with cholesterol to form a non-hemolytic compound. This reaction, in fact, is used as the basis of certain methods employed in the quantitative estimation of the cholesterol content of normal serum (4). The combination of cholesterol and digitonin is especially suited to a comparison of the degrees of neutralization of pneumococcus hemotoxin by normal and immune sera since Cole (1) has shown cholesterol to be one of the most important constituents involved in the inhibitory action of normal serum upon the pneumococcus lysin.

The experiments may be outlined as follows: The lytic action of a sample of digitonin was determined in the absence of serum. With this information at hand two dilutions of digitonin were prepared in salt solution; one solution contained in 1.5 cc. twice the amount required to hemolyze 2.5 cc. of a 1 per cent suspension of rabbit cells; the other solution contained four times that amount of the lytic agent. Mixtures of the digitonin with various dilutions of normal and immune rabbit serum were then prepared as described for the test mixture of pneumococcus hemotoxin and serum in the previous experiments. After incubation for 1 hour at 37°C. to allow time for the combination of digitonin with the cholesterol and other serum constituents, 0.5 cc. of a 5 per cent suspension of rabbit cells was added.

The results of these experiments were conclusive. Although relatively large amounts of both the normal and immune serum inhibited the digitonin hemolysis, there was no significant difference in the action of the two. The sera obtained by immunization with both the reduced and oxidized solutions exhibit more than 100 times the neutralizing capacity of normal serum when tested against pneumococcus hemotoxin, yet they exhibited no greater neutralizing or inhibiting action on digitonin hemolysis than did this serum. From the results, it is obvious that the neutralizing antibody produced during immunization with the hemolytically inactive, oxidized hemotoxin is distinct from the usual lysin-inhibiting constituents of normal serum.

Neutralization Experiments with Pneumococcus Immune Serum and Hemotoxins of Other Bacteria.

Experiments were conducted to determine whether the immune sera which neutralize pneumococcus hemotoxin have any neutralizing or inhibiting action upon the hemotoxins of other bacteria. Tetanolysin and the lysin of the Welch bacillus were the hemotoxins tested. Normal serum and the serum produced by immunization with heat-killed pneumococcus vaccine were included as controls, since certain constituents of normal serum have a marked inhibitory action upon the lysins of both of these anaerobic bacilli. The procedure employed was essentially the same as in the previous experiments with the pneumococcus lysin. The constant "dose" of tetanolysin and Welch lysin used in these experiments represents 8 to 10 times the amount of lysin required for the hemolysis of 2.5 cc. of rabbit cells in the absence of serum. A larger "dose" of lysin was chosen than in the previous experiments because these lysins are inhibited to a greater extent by normal serum than is the lysin of pneumococci. The preparation of serum-hemotoxin mixtures and other steps in the procedure were the same previously described.

Three normal sera and the sera of the four rabbits immunized with oxidized and with reduced pneumococcus extracts were included in the experiment. The results of tests upon tetanolysin with a typical normal and a typical immune serum are presented in Table III.

In spite of the markedly increased capacity of the immune serum to

neutralize the hemotoxin of Pneumococcus, the results presented in Table III which were typical for the experiments show that immunization of animals with this hemotoxin does not increase the ability of their serum to neutralize or inhibit the hemotoxin of the tetanus bacilli.

The results of the tests with the Welch lysin were not so clean-cut, since they were complicated by marked differences in the neutralizing or inhibiting capacity of different normal rabbit sera—a fact previously observed in another investigation (5). The sera of the various immune rabbits also showed marked differences among themselves in their inhibitory action on the Welch lysin. However, a survey of the results as a whole indicates quite clearly that the sera of rabbits

TABLE III.

Inhibition or Neutralization of Tetanolysin by Pneumococcus Immune Serum and by Normal Serum.

Serum	Hemolysis by previously incubated mixtures of serum and tetanolysin		
	Amount of serum		
	0.005 cc.	0.001 cc.	0.0002 cc.
Rabbit immunized against pneumococcus hemotoxin	0	+	+++
Normal rabbit	0	0	++

immunized against the hemotoxin of Pneumococcus exerted no greater neutralizing or inhibiting effect upon the lysin of Welch bacilli than was found with the serum of normal or non-immune animals.

COMMENT.

The preceding experiments indicate that a neutralizing antibody may be produced by immunization with the hemolytically inactive hemotoxin present in oxidized solutions as well as by immunization with the active hemotoxin. The antibody is a species-specific antihemotoxin neutralizing the hemotoxin from all types of pneumococci. It seems to be without effect upon the hemotoxins of tetanus and Welch bacilli.

The theoretical and practical importance of "toxoid immunization"

gives a special significance to the above results. But the data thus far procured do not enable one to state that the inactive or oxidized lysin possesses antigenic properties identical with those of the reduced or active hemotoxin. A more complete discussion of the antigenic properties of the inactive oxidation products of pneumococcus hemotoxin will be presented in a subsequent paper.

SUMMARY.

Immunization with solutions of the intracellular substances of pneumococci, in which the hemotoxin has been rendered inactive by oxidation, yields an antibody which neutralizes the active or reduced hemotoxin.

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