STUDIES ON IMMUNITY IN ANTHRAX

I. Variation in the Serum T-Agglutinin during Anthrax Infection in the Rabbit

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T-agglutinin is the term usually applied to the substance which is present in normal serum of many mammalian species and agglutinates erythrocytes whose receptors have been altered by the action of enzymes produced by certain bacteria (1, 2) or by viruses of the mumps-influenza group (3). Attention was first directed to the agglutinin by its ability to introduce complications in blood-typing procedures when the erythrocyte suspensions were altered by the action of certain contaminating bacteria (1). The observation that absorption and spontaneous elution of certain viruses produce similar agglutinability (3) stimulated further interest in the agglutinin. The T-agglutinin titer was increased above the normal in sera from primary atypical pneumonia when cold agglutinin was also elevated, although T-agglutinin was found to be distinct from cold agglutinin (4). No significant alteration in the T-agglutinin titer was observed, however, in human sera from mumps, influenza, psittacosis, or hepatitis (4).

In an effort to gain insight into some of the mechanisms which influence infection and immunity in anthrax, various serological and biochemical properties of the serum have been studied during the course of the infection. The mechanism of acquired immunity in anthrax appears to be related to that in typical virus infections, in that it is not associated with the activity of antibodies demonstrable by agglutination, precipitation, or complement fixation (5). It seemed possible, therefore, that alterations in cell receptors somewhat analagous to those in virus infections might take place and that these processes would be reflected in changes in tissue and serum factors associated with virus activity. The T-agglutinin titer was therefore studied during anthrax infection in the rabbit, and it was found that an abrupt decrease in titer frequently occurred during the acute phase of the infection. Observations on the nature and mechanism of this alteration in T-agglutinin titer are the subject of the present work.

Materials and Methods

The Vollum strain of *B. anthracis* was used. A spore suspension was prepared by growing the organism on nutrient agar for 3 days, harvesting in water, heating at 60°C. for 30 minutes

to destroy vegetative cells, and washing and resuspending in water. These suspensions have been stored in the refrigerator and have shown no apparent change in viable count or virulence after 2 years. Rabbits were challenged by intracutaneous injection on the back near the posterior axillary line. The receptor-destroying enzyme (R.D.E.) of *V. cholerae* was prepared by the method of Burnet and Stone (6), and stored at −20°C. in small tubes. No change in enzyme activity was noted after storage for 6 months under these conditions. The R.D.E.-treated erythrocyte suspension was prepared according to Lind and McArthur (4), using normal human type O erythrocytes. Rabbit serum samples were obtained from blood taken from the marginal ear vein or from the heart, and were stored at −20°C. until used. Sera from the various bleedings from each rabbit were titrated in the same series of tests. Sera were inactivated at 56°C. for 30 minutes before use.

Serial 2-fold dilutions of the sera were prepared in saline in a volume of 0.5 ml. and 0.5 ml. of 1 per cent R.D.E.-treated erythrocyte suspension added. The tests were incubated at 37°C. for a half hour, resuspended by shaking, and incubated for another half hour. They were then allowed to stand at room temperature for a half hour and read for the presence of macroscopic agglutination on resuspension. Titers are recorded as the final dilution showing definite evidence of agglutination.

EXPERIMENTAL RESULTS

A. Changes in T-Agglutinin Titer during Anthrax Infection.—T-agglutinin titers were determined on serum samples obtained from rabbits after intracutaneous challenge with 10^4 spores of *B. anthracis*. Most of these rabbits were being used for assay of the anthrax protective antigen (7) and therefore had received injections of various culture filtrates. The immunizing potency of the culture filtrates varied greatly, and accordingly the infections produced in the rabbits ranged from negative or minimal, as judged by the absence of any lesion at the challenge site, to the rapidly extending fatal infection characteristic of the normal animal (8). The challenge dose was invariably fatal for unprotected animals, and they usually died on the 3rd or 4th day after challenge. Serum samples were obtained before immunization, shortly before challenge, and at various intervals after challenge.

Typical results of T-agglutinin titrations on sera from two animals are given in detail in Table I. The approximate degree of agglutination observed on resuspending the settled erythrocytes is indicated by the number of plus marks. It will be observed that the T-agglutinin titer in rabbit 5-01 dropped to less than 1-8 on the 3rd day after challenge; the rabbit was found dead on the following day. Rabbit 3-77 had been immunized with an antigen preparation of moderate protective activity. Serum taken on the 5th day after challenge showed a marked decrease of T-agglutinin. The animal survived and the titer returned to normal on the 13th day after challenge. No significant change in T-agglutinin was noted as a result of the immunization procedures.

After the agglutination titrations had been recorded they were placed in the refrigerator and reexamined the following day. It was observed that some of the acute-phase sera produced partial hemolysis of the erythrocytes under these conditions, as shown in Table I. This reaction is discussed in part C.

TABLE I

T-Agglutination and Hemolysis with Sera Taken at Intervals before and after Challenge with B. anthracis

Rabbit No.	Serum sample	T-agglutination					Hemolysis*				
		Serum dilution					Serum dilution				
		1-8	1-16	1-32	1-64	1-128	1-8	1-16	1-32	1-64	1-128
5-01	Before immunization	++	+	+	−	−	±	−	−	−	−
	Before challenge	++	++	+	−	−	−	−	−	−	−
	Days after challenge: 3‡	−	−	−	−	−	++	++	++	++	+
3-77	Before immunization	++	++	+	+	+	−	−	−	−	−
	Before challenge	+++	++	+	+	+	+	−	−	−	−
	Days after challenge:										
	5	++	+	−	−	−	++	++	++	+	±
	9	++	++	+	+	−	−	−	−	−	−
	13	+++	++	++	+	+	−	−	−	−	−
	20	+++	+++	+	+	+	−	−	−		−

* After standing at 4°C. for 16 to 20 hours.

‡ Rabbit dead on 4th day after challenge.

TABLE II

Changes in T-Agglutinin Titer during Anthrax Infection in 95 Rabbits

Alteration in T-agglutinin titer during infection	No. of animals	Per cent of total animals tested	Survivors in group
		per cent	*per cent*
No significant change	36	38	36
Moderate decrease—less than 4-fold	26	27	35
Marked decrease—4-fold or greater	29	31	21
Increased	3	3	33
No agglutinin present	1	1	0

The results of T-agglutinin titrations on sera from 95 challenged animals are summarized in Table II, which records the number of animals which showed no change in titer, a moderate decrease of less than 4-fold, a marked decrease of 4-fold or greater, and an increase in titer. One rabbit was encountered whose

serum showed no agglutination at a dilution of 1-20. It will be observed that 58 per cent of the animals showed a significant decrease in titer during the infection. In 38 per cent of the animals no significant change in titer was observed. Several factors presumably contributed to the appreciable size of this latter group. Some of the animals were resistant to infection as a result of the immunization procedure, so that infection was absent or minimal in a number of them. The rabbits were bled in groups at rather wide time intervals, so that the time of bleeding was doubtless not optimum for the demonstration of a decrease in titer in many of the animals. In addition, the results are complicated somewhat by the presence of normal O-cell agglutinins in the sera of some of the rabbits. This factor would mask a decrease in T-agglutinin, especially when the normal T-titer was low.

T-agglutinin titers of sera from different normal rabbits varied from 1-8 or less to 1-128. Titers of successive bleedings from the same normal animal, however, were relatively constant. In animals which survived infection the T-agglutinin titer usually returned to the normal level, but not significantly above. Occasionally a 2-fold rise above the normal titer was observed, but the increase does not appear to be significant; evidently T-agglutinin is not directly involved in acquired immunity to anthrax.

B. Presence of a T-Agglutinin Inhibitor in Acute-Phase Sera.—The relatively abrupt decrease in T-agglutinin titer during the acute phase of the infection and its rapid return to normal during convalescence suggested that during the infection the serum acquired the ability to inhibit T-agglutination. Acute-phase sera were therefore tested for ability to inhibit the T-agglutinin of normal serum. Typical results with normal and acute-phase sera from four rabbits which had shown a marked decrease in T-agglutinin are given in Table III. It will be observed that acute-phase sera, but not normal sera, were capable of inhibiting the T-agglutinin of normal serum. Of the 29 acute-phase sera which had shown a markedly reduced titer of T-agglutinin 26 were examined for the presence of inhibitor of T-agglutinin; 20 were found to have detectable inhibitory activity, varying from partial inhibition at a dilution of 1-8 to almost complete inhibition at 1-128. The pattern of the inhibition effect varied considerably with sera from different animals, as is shown by the examples presented in Table III. With some sera the inhibition was complete in low dilutions and abruptly disappeared in higher dilutions. In others partial inhibition appeared over a considerable range of dilutions, and a quantitative end-point was therefore difficult to establish. The present data suggest no explanation for this variability.

The substance responsible for inhibition of T-agglutination did not appear to be related to the polypeptide (9) or polysaccharide (10) antigens of *B. anthracis*, since it was found that as much as 0.1 mg. of these substances did not produce detectable inhibition of T-agglutination. Culture filtrates containing anthrax protective antigen (7) and edema fluid from cutaneous anthrax

lesions in the rabbit (11) were also devoid of inhibitory activity. The substance in acute-phase sera responsible for inhibition of T-agglutination will therefore be referred to as T-inhibitor.

Although T-agglutinin was not observed to increase significantly above the normal level on recovery from the infection, it seemed possible that the serum might acquire some new ability to neutralize T-inhibitor. This possibility was tested by substituting convalescent sera for normal serum in the T-inhibition titration. The convalescent sera were diluted so that, as with the normal serum, two agglutinating doses of serum were added to each tube. The inhibition titers

TABLE III

Effect of Normal and Acute-Phase Sera on T-Agglutination

Rabbit serum tested	Final serum dilution				
	1-8	1-16	1-32	1-64	1-128
3-66 before infection	++	++	++	++	++
Acute phase	−	−	±	+	++
3-71 before infection	+++	++	++	++	++
Acute phase	−	±	±	±	+
3-91 before infection	+++	+++	+++	++	++
Acute phase	±	±	+	++	++
4-96 before infection	+++	++	++	++	++
Acute phase	+	+	++	++	++

Dilutions of the test sera in a volume of 0.25 ml. were mixed with an equal volume of normal serum diluted to contain two agglutinating units and incubated for 1 hour at 37°C.; 0.5 ml. of R.D.E.-treated erthrocyte suspension was then added, and the tests incubated and read for T-agglutination.

of acute-phase sera were not found to change appreciably when convalescent serum was substituted for normal serum in the titration. This suggests that no increased ability to neutralize T-inhibitor accompanies acquired immunity to anthrax.

C. Hemolytic Activity of Acute-Phase Sera.—In the course of titrations of T-agglutinin it was observed that those acute-phase sera in which a marked decrease in T-agglutinin had occurred and in which excess T-inhibitor could be demonstrated usually produced definite hemolysis when the titrations were left at 4°C. overnight. Examples of this behavior are given in Table I. Of 20 sera found to contain T-inhibitor, 19 showed appreciable hemolytic activity. The intensity of hemolysis varied considerably, but was usually not complete even at 1-8, the lowest dilution tested.

Since the sera were inactivated for 30 minutes at 56°C. before titration, the reaction apparently did not require the presence of labile components of complement. Inactivation of fresh serum at 56°C. for 10 minutes was found to produce an abrupt decrease in hemolytic activity; with normal sera or with sera devoid of T-inhibitor this treatment essentially destroyed hemolytic activity. Part of the hemolytic activity of acute-phase sera was also destroyed by this treatment, but the residual activity did not show appreciable further decrease after 1 hour at 56°C. In general the heat-stable hemolytic activity of acute-phase sera was correlated with the T-inhibitor activity, suggesting that the two effects may be produced by the same substance.

Hemolytic activity was found to be inhibited in the presence of 0.05 M sodium citrate; addition of calcium or magnesium ion did not, however, increase the hemolytic activity in the absence of citrate. Untreated human type 0 erythrocytes were hemolyzed approximately as readily as were R.D.E.-treated erythrocytes. Untreated erythrocytes of other species gave irregular results with different hemolytic sera. Of 6 sera tested, 3 lysed rabbit erythrocytes nearly as well as human erythrocytes; 1 serum also lysed sheep erythrocytes.

D. In Vitro Changes in T-Agglutinin and T-Inhibitor Titers.—In the course of the work on T-agglutinin and T-inhibitor repeated titrations were frequently carried out on the same serum samples. While the agreement of these titrations was reasonably good, definite decreases in T-agglutinin were occasionally encountered on retitration of acute-phase sera. The T-agglutinin titers of normal sera, however, were relatively constant. Although the sera were stored in the frozen state, they were occasionally thawed to remove small samples for these and other experiments, and were thus allowed to stand at room temperature for short intervals. It seemed possible, therefore, that T-inhibitor activity could appear in acute-phase sera *in vitro*. Preliminary experiments have shown that this can occur.

Sera were incubated at 37°C. for various periods up to 20 hours at a dilution of 1-2 in saline which contained 1-5000 merthiolate. Merthiolate was included to prevent bacterial growth during the longer incubation times; its addition did not influence the reaction being studied. A number of freshly drawn acute-phase sera were observed to show a gradual decrease in T-agglutinin titer or an increase in T-inhibitor titer and an increase in hemolytic activity on incubation at 37°C. Normal sera also showed decreases in T-agglutinin titer, but the changes were much less marked than with acute-phase sera. Further investigations on the *in vitro* production of T-inhibitory activity are in progress.

DISCUSSION

It is not clear from present evidence what relationship production of the T-inhibitor has to the fundamental processes of anthrax infection. It is possible that the mechanism which leads to production of T-inhibitor is a secondary and

non-specific response to acute infection. The hemolytic activity which appears to be associated with the presence of T-inhibitor, however, suggests that the substance is not without significance in the disease process; present evidence is not sufficient to determine whether it plays a role in the mechanism of death in anthrax.

The factors studied appear to bear no direct relationship to the immune response to anthrax infection. The T-agglutinin titer returns to normal on recovery, but has not been observed to increase significantly above the initial level. T-inhibitor would not appear to be identical with the protective antigen or the known serologically active antigens of *B. anthracis*. Convalescent serum appears to contain no neutralizing capacity for T-inhibitor in addition to its T-agglutinin activity. It seems possible, however, that immunity may involve suppression of those *in vivo* processes which lead to the production of T-inhibitor; experiments suggested by this hypothesis are in progress.

Various findings suggest that the major phenomena under investigation are influenced and complicated by variables whose nature and precise effect are unknown at present. Thus the variation in the sharpness of the end-point in inhibition experiments suggests that some factor other than the concentration of the inhibitory substance plays a role in determining its effectiveness in inhibiting T-agglutination. The observed variation in the hemolytic activity of different sera on erythrocytes of different species is also unexplained, although it may be related to the variation of normal agglutinin for the erythrocytes in the sera; preliminary data are compatible with the hypothesis that hemolysis of human and sheep erythrocytes is inhibited by the presence of normal agglutinin. It seems reasonable that the decrease in T-agglutinin is in some cases masked by the presence of normal antihuman agglutinins, since R.D.E.-treated erythrocytes retain their normal antigenic specificities to an appreciable extent (12). In the present study we have investigated only what appeared to be the major and most general changes and effects; more detailed work can doubtless provide explanations for some of the more complex observations.

SUMMARY

An abrupt decrease in the titer of serum T-agglutinin frequently occurs during the acute phase of anthrax infection in the rabbit. In partially immunized animals which survive the infection the titer returns to normal during convalescence, but has not been observed to rise significantly above the normal level.

The presence of a substance capable of inhibiting T-agglutination may be demonstrated in the sera of many of those animals in which a marked decrease in T-agglutinin has occurred. The polysaccharide and polypeptide antigens of *B. anthracis* do not possess this activity.

Sera containing the T-inhibitory substance usually produce a slow hemolysis of T-erythrocytes and also of normal human type O erythrocytes; hemolysis of

erythrocytes of other species is irregular. This reactivity of the sera withstands inactivation at 56°C. but is inhibited by citrate.

T-inhibitory and hemolytic activities can frequently be increased by incubation of the acute-phase serum at 37°C.

The ability of normal serum to neutralize the T-inhibitory and hemolytic activities of acute-phase serum is not significantly increased after recovery from the infection.

The meaning of the results is discussed.

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