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Re-Examination of the Neisser–Wechsberg (Antibody Prozone) Phenomenon

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Summary. Although the Neisser–Wechsberg phenomenon resembles the inhibition of agglutination systems by excess antibody, the experimental results have indicated that with heat-inactivated antiserum the phenomenon results both from an excess of specific antibody and of non-specific anti-complementary activity. Complement (C) has been shown to be fixed in the presence of the excess antiserum which inhibits the bactericidal reaction. The inhibition was overcome by an excess of the third complement component factors indicating that the excess of antiserum interfered with the activation or function of the components acting at one of the late steps in the reaction sequence. The prozone phenomenon was relatively slight when unheated antiserum was used or when sensitized organisms were washed to remove serum substances unrelated to antibody. Non-specific anti-complementary activity, therefore, is a major contributor to the prozone phenomenon. Both IgM and IgG fractions of rabbit antisera elicited a prozone although the former had relatively greater bactericidal than inhibitory activity.

INTRODUCTION

The Neisser–Wechsberg phenomenon relates to the inhibition by excess antiserum of the bactericidal reaction mediated by the complement (C) system (Neisser and Wechsberg, 1901). This inhibition by excess antiserum may occur when the normal serum serving as a C source is either lacking in bactericidal activity itself or capable of limited activity because of the presence of normal antibody. In the latter situation, an excess of antiserum may annul the normal serum's bactericidal effect. The Neisser–Wechsberg effect has never been satisfactorily explained, although it has been regarded as a zone phenomenon analogous to that seen in bacterial agglutination (Wilson and Miles, 1964). When antibody molecules are in great excess relative to the number of functional groups on cells, the simultaneous attachment of both sites of individual multivalent antibody molecules to two cells would be improbable, and agglutination would not result. The bactericidal antibody prozone phenomenon may occur, however, in the presence of gross agglutination by large amounts of antiserum and also without detectable agglutination with smaller amounts. Moreover, in agglutination reactions the prozone phenomenon may not be related simply to antibody excess, but often involves a special class of 'incomplete' or 'blocking' antibody, which combines with cellular antigens, but does not agglutinate the cells. Comparable non-complement-fixing antibodies may of course be

involved in the prozone seen with bactericidal antibodies, but they have not been demonstrated. Finally, serum C does not play a part in the agglutination reaction, but its inactivation by antiserum may be significantly involved in the prozone of bactericidal antibodies. Similar considerations apply to the inhibition by excess antiserum of the haemolytic reaction mediated by the C system. The present investigation was designed to re-evaluate certain of the older observations relating to this antibody prozone phenomenon and to attempt several new approaches to aid in its understanding.

MATERIALS AND METHODS

Serological reagents

Most of the bactericidal tests were performed with a rabbit antiserum obtained after eight intravenous injections at weekly intervals with about 1×10^8 cells of heat-killed *Salmonella typhi* O 901. The animal was bled 1 week after the last injection. Lyophilized guinea-pig serum, product of Texas Biological Laboratories, Fort Worth, reconstituted with water prior to use, served as a C source.

Immune bactericidal reaction

A quantitative photometric assay with *S. typhi* O 901 as the test organism was used (Muschel and Treffers, 1956). When the reaction mixtures were shaken during the 1-hour reaction period to minimize agglutination, the tubes were placed in a constant temperature (37°) water bath agitated at about 210 vibrations/min.

Immune haemolysis

These reactions were performed by a standardized quantitative procedure (Muschel and Lowe, 1955).

Immune adherence

The immune adherence procedure was a modification of the method of Nishioka (1963). Three-quarters of a millilitre of a 3 per cent suspension of sheep red blood cells and an equal volume of the haemolysin dilution were incubated together for 10 minutes at 4°, then 3·0 ml of veronal buffer diluent and 3·0 ml of 1 : 550 guinea-pig C or heat inactivated C as a control, were added, and the mixtures incubated at 37° for 1 hour. To a 0·5-ml sample of these mixtures, 0·4 ml of veronal buffer and 0·1 ml of 1·5 per cent human group O, Rh_0 (D) negative red cells were added. After a further incubation at 37°, adherence of the sheep cells to the human cells was noted both by the red cell pattern and by microscopic examination.

Immunoconglutinin

Immunoconglutinin was estimated by the method of Coombs, Coombs and Ingram (1961) and absorbed from serum by the procedure of Ingram (1959).

Serum fractions

The IgM and IgG fractions of the rabbit antiserum were separated by gel filtration (Borsos and Rapp, 1965) by T. Borsos (National Institutes of Health). Separation by centrifugal force was carried out by diluting the serum samples with an equal volume of the saline diluent used in the bactericidal test (Muschel and Treffers, 1956), centrifuging at

40,000 rev/min for 7 hours at 4° in a Beckman No. 40 rotor with tubes containing 12 ml of the serum dilutions (Trautman and Cowan, 1968) and separating into seven equal fractions. The separations were kindly carried out by Mr David Peterson of this department.

RESULTS

INHIBITION OF THE ANTIBODY PROZONE BY EXCESS C

One of the most significant observations about the bactericidal antibody prozone is that it is favoured by limiting amounts of C and may be overcome by greater amounts (Table 1). This result suggests that the prozone phenomenon results from an anti-complementary effect of the antiserum. Apparently the antiserum may inactivate C, prevent its activation, or sterically interfere with its activity.

TABLE 1

THE REVERSAL OF THE ANTIBODY PROZONE IN THE IMMUNE BACTERICIDAL REACTION BY ADDITIONAL GUINEA-PIG C

Heated immune serum (ml)	Percentage of bacteria killed (ml guinea-pig C)			
	0·025	0·05	0·1	0·2
0·2 of 1 : 25	0	0	34	94
0·2 of 1 : 100	46	90	100	100
0·2 of 1 : 400	88	98	100	100
0·2 of 1 : 1600	86	99	100	100

Test organism: *S. typhi* O 901. The 0·05 ml and greater amounts of the C itself killed over 90 per cent and 0·025 ml about 50 per cent of the bacteria.

RESPONSIBILITY OF ANTIBODY FOR THE PROZONE

An obvious possible explanation involves the inhibition of bactericidal activity and of haemolysis as a result of agglutination. However, in agreement with the results of others (Buxton, 1905), we have found that one sheep red cell antiserum having an agglutination titre of 1 : 640 did not manifest the prozone phenomenon. Under identical test conditions, another antiserum with a similar agglutination titre showed a marked prozone reaction with 85 per cent haemolysis when the serum was diluted 1 : 1024 and no detectable reaction at a 1 : 4 dilution. Moreover, in the bactericidal reaction, the prozone can be demonstrated with amounts of antiserum that do not give any visible agglutination. Finally, when the bactericidal reaction was performed with vigorous shaking to diminish agglutination, there was no reduction in the prozone.

The antibody prozone has been generally observed with heat inactivated antiserum. Normal heated rabbit serum may be non-specifically anti-complementary so that it was of importance to determine the existence of the antibody prozone with active serum. Although considerably diminished, the prozone definitely occurs with active serum (Table 2). One notes that with the largest amount of untreated antiserum tested (0·2 ml of 1 : 5 dilution) a definitive prozone was not observed, whereas the next smallest volume (0·2 ml of 1 : 25 dilution) gave a small but definite inhibition of the bactericidal reaction. This experiment was repeated and the results confirmed. Conceivably, this finding was elicited by the C contribution of untreated antisera which tends to annul the prozone effect with the largest volume of the antiserum.

TABLE 2

BACTERICIDAL REACTION WITH A HEATED (56°, 30 MINUTES) AND UNHEATED SAMPLE OF ANTISERUM AGAINST *S. typhi* O 901

Antiserum (ml)	Percentage of bacteria killed	
	Unheated	Heated
0·2 of 1 : 5	93	0
0·2 of 1 : 25	65	69
0·2 of 1 : 125	93	94
0·2 of 1 : 625	96	96

C source: 0·025 ml of guinea-pig serum.

To investigate further the possibility that antibody is at least partly responsible for the prozone phenomenon, different amounts of antiserum were added to bacterial cells suspended in diluent and the mixtures allowed to incubate for 1 hour at 23°. After centrifugation, the supernatants were removed so that most or all serum substances unrelated to antibody were not present during the bactericidal reaction after the addition of C. Controls were included that were treated in the same way except that the supernatant was not removed. The results indicated that, although of lesser magnitude, a prozone was produced with a large amount of serum (0·4 ml) when the supernatant was removed (Table 3). In addition, an antiserum against *S. typhi* O 901, capable of a marked prozone reaction against that organism, was only slightly more inhibitory than the pre-immunization control serum in the standard immune haemolytic system. This result suggests that the disproportionately greater prozone reaction of the antiserum against its homologous antigen may be attributed to its antibody content. Antibody *per se*, therefore, may produce a prozone augmented however by other non-specific substances.

TABLE 3

THE EFFECT OF REMOVAL OF SUPERNATANT PRIOR TO THE ADDITION OF C UPON THE REACTION AGAINST CELLS OF *S. typhi* O 901 SENSITIZED BY DIFFERENT AMOUNTS OF ANTISERUM

Rabbit antiserum (ml)	Percentage of bacteria killed	
	Supernatant removed	Supernatant not removed
0·4	44	0
0·004	85	91
None*	23	16

*C alone: 0·025 ml guinea-pig serum.

C-FIXATION AND THE ANTIBODY PROZONE

Experiments were performed to determine if C was fixed in the presence of the excessive amounts of antiserum that lead to a prozone. In one of these experiments, after the reaction period of 1 hour at 37° with different amounts of antiserum and constant volumes of C and test organisms, the C content of the fluid phase was assayed. With an amount of antiserum that did not kill any bacteria and 1/64th of that amount that resulted in 87 per cent killing, C assays indicated that over twice as much haemolytic C was recovered in the supernatant of the tubes with the bactericidal amount of antiserum compared with the amount remaining in the tubes with the prozone amount. C was fixed or destroyed, therefore, with prozone amounts of the antiserum.

The next experiment using the technique of immune adherence was designed to determine if the fixed C was present on the cell surface. Because of technical difficulty with the use of bacteria as a test antigen in immune adherence, the red cell system was used. A positive immune adherence result with an excessive amount of haemolysin that inhibited haemolysis indicated that some of the C3 of guinea-pig C (Müller-Eberhard, 1968) was present on the red cell surface despite the cell's resistance to haemolysis. A control tube with inactivated C gave a negative result.

Another haemolytic experiment was performed to determine the step in the C sequence in which an excess of antiserum led to an inhibition of the reaction. After incubation at 37° for 1 hour, 60 per cent haemolysis was obtained with a moderate amount of haemolysin (1 : 256 dilution) and a limited amount of guinea-pig serum as a C source and only 20 per cent with an excessive amount of haemolysin (1 : 8 dilution). After centrifugation and removal of the supernatant, C-EDTA (guinea-pig serum diluted 1 : 200 in 0·85 per cent NaCl with 0·002 M EDTA) was added to the unlysed red cells. C-EDTA provides the C3 factors (C3,5,6,7,8,9) and lyses cells in the EAC1a,4,2a state (Mayer, 1965). Unexpectedly, the surviving red cells sensitized with the larger, prozone amount of haemolysin were considerably more sensitive to C-EDTA and 40 per cent of these cells were lysed in contrast to no lysis of the surviving cells sensitized with less haemolysin. An excess of the C3 factors, therefore, may overcome the antiserum prozone.

TESTS WITH SERUM FRACTIONS

An obvious possibility to account for the antibody prozone is the presence of competitive non-complement-fixing antibodies in rabbit antiserum. Bactericidal tests were performed with IgM and IgG fractions of a potent antiserum. Both fractions were capable of exerting a bactericidal effect and both showed an antibody prozone. Fractions of a potent antiserum obtained by centrifugation were also tested. All of the fractions except the two lightest demonstrated considerable bactericidal antibody levels and detectable prozoning (Table 4). Each of the two lightest fractions showed only slight bactericidal antibody

TABLE 4

BACTERICIDAL ANTIBODY AND PROZONE TITRES OF ANTISERUM FRACTIONS SEPARATED BY CENTRIFUGATION AGAINST *S. typhi* O 901

Fraction*	IgG concentration†	Bactericidal antibody titre	Prozone titre‡	Antibody/prozone ratio
1	ND	102	Less than 25	
2	ND	125	Less than 25	
3	5·0	1,430	161	8·9
4	3·0	2,780	370	7·5
5	2·0	2,000	360	5·6
6	1·3	7,700	1,080	7·0
7	1·0	288,000	4,360	66·0
Whole serum (unfractionated)	ND	37,000	770	48·0

ND, Not done.

* Fraction 1 represents the uppermost lightest fraction after centrifugation and fraction 7 the lowest and heaviest fraction containing the pellet. The fractions and the whole antiserum were heat inactivated prior to testing.

† Estimate of the relative amount of IgG based upon the amount of precipitate in millimetres formed with 2 cm of antiserum and 2 cm of the fraction in a capillary tube (diameter 1·0 mm) after 18 hours incubation at 4°.

‡ Represents the amount of serum or serum fraction required to give killing of 50 per cent of the bacteria in the range where decreasing amounts of antiserum give a greater percentage killed.

activity approximately equal to that of whole untreated normal serum. These experiments obviously did not reveal any unique non-complement-fixing antibody in the fractions tested. Both the greatest bactericidal activity and the greatest inhibitory activity were found in the heaviest fraction containing most or all of the IgM serum fraction and the least amount of IgG. Although the heaviest fraction (fraction 7) contained almost 150 times as much bactericidal antibody as fraction 5, the prozoning potency of fraction 7 was only about twelve times as great as that of fraction 5. There is also a lack of correlation between bactericidal antibody titres and the prozone with unfractionated antisera. For example, one antiserum resulting from purified endotoxin injections gave a bactericidal antibody titre of 50,000, but its prozone titre was not greater than 5, whereas another antiserum elicited by whole bacterial cells gave a bactericidal antibody titre of 65,000 and prozone titre of about 60. Bactericidal antibody alone, therefore, cannot be equated with the prozone.

COMPETITION BETWEEN THE BACTERIAL CELL AND SOLUBLE ANTIGEN FOR ANTIBODY

Another explanation recently proposed to account for the prozone is the presence in the reaction mixtures of soluble antigen, which may compete with the antigens of the bacterial cell for antibody (Coombs, 1965). C-fixation may occur, accordingly at an ineffective site. Many experiments were performed to test this thesis, but they all revealed that under the standardized test conditions the extent of such fixation was negligible. In the first place, when the tests were performed with washed organisms suspended in saline diluent instead of broth, the extent of the prozone was not diminished. In addition, an increase in the amount of soluble antigen in the reaction mixture did not appreciably affect the extent of the prozone. When 1·4 ml of the supernatant of a culture grown under the usual test conditions, but without serum substances, was added to the reaction mixture in place of the saline diluent, there was no effect with the larger amounts of antiserum exerting a prozone reaction (Table 5). However, with a higher dilution of the antiserum (1 : 1600), the supernatant was inhibitory (31 per cent of bacteria killed against 76 per cent for the control) probably as a result of competition with the antigens of the cell surface for the limited amount of antibody.

TABLE 5

EFFECT OF CULTURE SUPERNATANT ON THE BACTERICIDAL REACTION AGAINST *S. typhi* O 901

Antiserum (0·2 ml of dilution)	Percentage of organisms killed	
	With extra supernatant*	Control
1 : 25	0	0
1 : 50	0	0
1 : 100	28	23
1 : 200	75	74
1 : 400	91	93
1 : 800	71	93
1 : 1600	31	76

0·025 ml guinea-pig C added to all tests.

* 1·4 ml of supernatant of the culture under test conditions without serum substances was added to the reaction mixtures in place of the saline diluent in the control.

IMMUNOCONGLUTININ

Since rabbit antiserum against bacteria demonstrates elevated levels of immunoconglutinin (Coombs *et al.*, 1961), and since immunoconglutinin may exert an anti-complementary effect, it seemed likely that the non-specific anti-complementary effect of antisera might be associated with immunoconglutinin. Accordingly, a sample of antiserum exerting a prozone and containing easily detectable immunoconglutinin was absorbed with sensitized sheep red cells alexinated with horse-serum, another sample of the antiserum absorbed with sensitized shep red cells, and a third sample was untreated (Ingram, 1959). Although removal of detectable conglutinin was accomplished by the alexinated sensitized sheep cells, there was no detectable effect on the prozone in the bactericidal reaction, which was performed, with horse C in place of guinea-pig C because of possible differences in the reactivity of immunoconglutinin with C components of different species.

DISCUSSION

The results of this study suggest that the Neisser–Wechsberg phenomenon results both from an excess of specific antibody and of non-specific anti-complementary activity of heat inactivated antiserum. That an excess of antibody per se may give rise to the prozone phenomenon was demonstrated by the occurrence of the prozone with unheated antisera (Table 2) and with organisms sensitized with heated antisera and then washed to remove non-antibody serum substances (Table 3). Neisser and Wechsberg's explanation of the zone phenomenon which they discovered was based upon Ehrlich's supposition of a complementophile group of the amboceptors and the absorption and resulting inactivation of C by the excess of amboceptor that had not united with the bacteria (Zinsser, Enders and Fothergill, 1935). The excess of antibody was ineffective, therefore, and led to C deviation. However, the specificity of the antibody prozone, as indicated in our experimental results, partly refutes this explanation. Yet the rather marked contribution to the antibody prozone by non-specific anti-complementary activity (Tables 2 and 3) produced probably by elevated levels of globulin is in accord with the original explanation.

The results indicated that the non-specific anti-complementary activity of heated antiserum, in addition to antibody, contributed substantially to the Neisser–Wechsberg effect. Even normal heat inactivated serum may be anti-complementary and this activity may be enhanced in the serum of immunized animals. It is well known too that sera from human patients with microbial infections and elevated γ-globulin levels are more likely to be anti-complementary than normal control sera (Davis, Kabat, Harris and Moore, 1944).

Immunoconglutinin, which seemed likely to account for the increased anticomplementary activity of antisera, did not contribute significantly, however, to the prozone effect with the sera tested. The substance or substances responsible for the anti-complementary activity in antisera, therefore, have not been identified. Another likely possibility is increased levels of γ-globulin, which upon aggregation by heating become markedly anti-complementary (Osler, 1961).

No evidence was obtained that competitive non-complement fixing antibodies were involved in the antibody prozone. All the antiserum fractions tested contained both bactericidal antibody activity as well as inhibitory activity. Similar results have recently been obtained by others (Daguillard and Edsall, 1968). It is noteworthy that the heavy 19S

fraction had the greatest ratio of antibody to prozone activity (Table 4) so that, if non-complement fixing antibodies contributed to the prozone, they were concentrated in the lighter molecules.

Despite the lack of a bactericidal or haemolytic effect with excess antiserum, C was shown to be fixed and C3 present on the red cell surface as indicated by a positive result in immune adherence (Müller-Eberhard, 1968). Quantitation is difficult, however, with the immune adherence technique and it is possible that less C3 is present on a red cell surface with excess antiserum. In addition, since an excess of C3 and the late reacting components annulled the prozone phenomenon, an excess of antiserum does not interfere, therefore, with the early steps in the immune haemolytic, and presumably bactericidal, reaction involving the activation and function of the C1, C4 and C2 components.

Finally, the possibility of C fixation at an ineffective site as a result of the release of antigen from the bacterial surface was considered. Theoretically such antigen could divert excess antibody from the cell surface and contribute to the prozone effect. It was found, however, that under the usual experimental conditions, the amount of such antigen did not exert a detectable effect in the antibody excess region. The occurrence of the prozone in the immune haemolytic reaction with washed red cells, from which little or no antigen was liberated is also in accord with the bactericidal reaction results.

The significance of the antibody prozone remains to be considered. Although the possibility exists that a prozone effect may arise as a result of hyperimmunization (Daguillard and Edsall, 1968), the limited extent of the prozone with active antiserum, demonstrable only with limited amounts of C (0·025 ml, Table 2), makes it unlikely that the prozone phenomenon occurs *in vivo* with undiluted plasma. There would seem to be little basis for concern, therefore, that the protection given by the C system may be jeopardized by high levels of antibody in individuals deliberately immunized or infected with C susceptible organisms. Yet it is well to be congnizant of the antibody prozone, and further studies particularly with active sera from humans immunized with vaccines of the Gram-negative bacteria would be desirable.

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