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BLOCKING ANTIBODIES SPECIFIC FOR HUMAN ALBUMIN INTERFERE WITH THE HEMOLYTIC ACTIVITY OF THE MEMBRANE ATTACK COMPLEX OF COMPLEMENT[1]

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Earlier, we showed that antibodies specific for human serum albumin are able to bind to erythrocyte-associated albumin and inhibit complement mediated hemolysis. In the present study we determine if inhibition is occurring at the membrane attack phase of complement activation or at an earlier step. We show that although cell-bound anti-albumin antibodies do not inhibit binding and activation of C3 or uptake of C9, they do appear to cause cells to become refractory to lysis by the membrane attack complex as they inhibit both the kinetics and the extent of hemolysis in a reactive lysis system which employs preformed C5b6 plus C7, C8, and C9. We believe that this is the first report of inhibition of the hemolytic activity of the membrane attack complex by antibodies bound to an erythrocyte surface antigen.

Antibodies, after binding to cell surface antigens, usually activate complement leading to opsonization by C3b and formation of the membrane attack complex, consisting of C5b-C9 on the cell membrane. Complement activation is one of the primary roles of antibodies in the humoral immune response. However, some bacteria, by generating "blocking" antibodies are able to circumvent destruction by the membrane attack complex. For example, blocking antibodies that bind to Neisseria gonorrhoeae and inhibit complement killing are found in the sera of many humans. Invesitgators were surprised to find that these antibodies increase, rather than decrease, the deposition of complement components on the cell surface (1).

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Abbreviations used in this paper: LPS, lipopolysaccharide; E-LPS-AB, erythrocytes coated with B. abortus LPS and sensitized with antibodies to the LPS; GVB, Veronal buffered sodium chloride containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin; EDTA-GVB, 40 mM EDTA in gelatin Veronal buffer; C-EDTA, serum complement diluted 1:10 with EDTA-GVB; HSA, human serum albumin.

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Earlier, we reported that polyclonal antibodies to human serum albumin bind to albumin, naturally occurring on human erythrocytes, and act as blocking antibodies (2). Although, the anti-albumin antibodies activate complement, lysis does not occur. In addition, these antibodies inhibit complement-mediated hemolysis by other "lytic" antibodies when both are bound to the cell.

In this study, we show that anti-albumin antibodies do not block hemolysis by inhibiting an intermediate step in the complement cascade. Rather, these antibodies appear to decrease the lytic capability of the membrane attack complex without prohibiting its formation on the red blood cell surface. We believe that this is the first report of inhibition of membrane attack complex hemolytic activity by an antibody bound to an erythrocyte surface antigen. Others have noted that in cases of autoimmune hemolytic anemia different patients with similar amounts of red blood cell bound IgG (even of the same subclass) and/or complement may suffer severe hemolysis or have normal red blood cell survivial (reviewed in 3). The reasons for this paradox are unknown. Perhaps antibodies to erythrocytes occur that are analogous to blocking antibodies involved in complement mediated killing of bacteria.

MATERIALS AND METHODS

Erythrocytes were isolated from whole blood drawn into either modified Alsevers solution or citrate-phosphate-dextose-adenine as anticoagulants. The blood was stored at 4° to 7°C and used within one month.

Antisera. Goat polyclonal anti-human serum albumin (HSA) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and was heat inactivated (56°C/60 min) shortly before use. Rabbit anti-goat IgG conjugated to peroxidase was obtained from Sigma Chemical Co. (St. Louis, MO). Bovine antibodies specific for Brucella abortus were acquired as described (4) from cattle testing positive for brucellosis. Goat anti-human C3 and goat anti-human C9 were purchased from Quidel (San Diego, CA).

Lipopolysaccharide (LPS) antigen was extracted from Brucella abortus as previously described (4).

Isotonic buffer solutions. The preparations of PBS, Veronal buffered sodium chloride containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% gelatin (GVB), and 40 mM EDTA in gelatin Veronal buffer (EDTA-GVB), have been described (4).

Complement and complement components. Human serum was obtained from clotted whole blood after centrifugation and stored at -70°C. Human C5b6 was prepared according to the method of Yamamoto and Gewurz (5). Human C7 and C8 were purchased from Quidel (San Diego, CA). C9 was prepared by the method of Biesecker et al. (6). Human serum diluted 1:10 with EDTA-GVB (C-EDTA) was used as a source of C8 and C9 in some experiments.

Sensitization of erythrocytes. Human erythrocytes , prepared for use in a passive lysis system, were coated with optimal amounts of B. abortus LPS and sensitized with optimal concentrations of antibody to LPS (E-LPS-Ab) using previously published procedures (7).

Detection of erythrocyte-bound complement components by immunoblot assays. Both human erythrocytes and human E-LPS-Ab (5×10^7 cells/ml) were incubated with

either goat anti-HSA (1:25) or GVB as a control for 30 min at 37°C. The cells were washed three times with PBS, one time with GVB, and resuspended at 5 x 10^7 cells/ml. All cell preparations were then treated with human complement (1:10) for 60 min at 37°C and the percent lysis was determined. The lysed and unlysed cells were pelleted together, washed two times with GVB and two times with 5 mM phosphate buffer, pH 8.0. The cell membranes were solubilized with SDS in the presence of 5% 2-mercaptoethanol and electrophoresed on 7.5% polyacrylamide gels as described by Laemmli (8) using the BioRad minigel system. The separated proteins were then transferred to nitrocellulose sheets (100 V for 60 min) according to the procedure of Towbin (9). Cell associated complement proteins were visualized using 5% nonfat dry milk in PBS as a blocking agent, with goat anti-C3 or anti-C9, and peroxidase conjugated anti-goat IgG.

Reactive lysis inhibition assay. Washed human erythrocytes (2 X 10^8 cells/ml in GVB without CA++ and Mg++) were incubated with goat anti-HSA (1:10), and control erythrocytes were incubated with GVB for 30 min at 37°C. After the erythrocytes were washed, 2 X 10^8 cells were pelleted and the supernate was removed. Excess C5b6 was added to each cell pellet and incubated for 10 min at 37°C. Then 200 ng C7 were added and the incubation was continued for another 10 min at 37°C. The cells were washed three times with GVB and resuspended at 1 X 10^8 cells/ml. Human serum, diluted 1:10 with EDTA-GVB, as a source of C8 and C9 was added to one volume of cells. Alternatively, purified C8 and C9, diluted to 1 μg/ml and 3 μg/ml respectively, were added instead of C-EDTA. The mixtures were incubated at 37°C for 60 min except in the kinetic study where duplicate 0.15 ml aliquots were withdrawn at various time points. Percent lysis was determined for each sample by measuring hemoglobin release spectrophotometrically.

RESULTS AND DISCUSSION

As we showed in an earlier publication (2), cell bound antibodies to HSA not only fail to support complement-mediated lysis but their presence on erythrocytes can even inhibit complement-mediated hemolysis initiated by "lytic" antibodies. Erythrocytes that bear only anti-albumin antibodies are not lysed by complement. In addition, albumin specific antibodies when bound to erythrocytes render them less susceptible to classical pathway mediated lysis when a passive lysis system is used. The anti-albumin antibodies caused a decrease in lysis from 57% to 22%. We utilized the passive lysis system in earlier work regarding interactions of complement with cell surfaces (2,4,10). B. abortus LPS, unlike LPS of enteric bacteria, activates neither the classical nor the alternative pathway of complement (7). One major advantage of this system is that the complement activating antibodies bind to a passively applied foreign antigen and, therefore, are not likely to block access of particular cell surface constituents to serum proteins. The intense agglutination frequently resulting from antibodies directed against human erythrocyte surface antigens is also avoided.

Although cell-bound anti-HSA inhibits the ability of complement to lyse erythrocytes it does not appear to accomplish this by stopping complement activation at an intermediate step in the cascade or by preventing association of complement

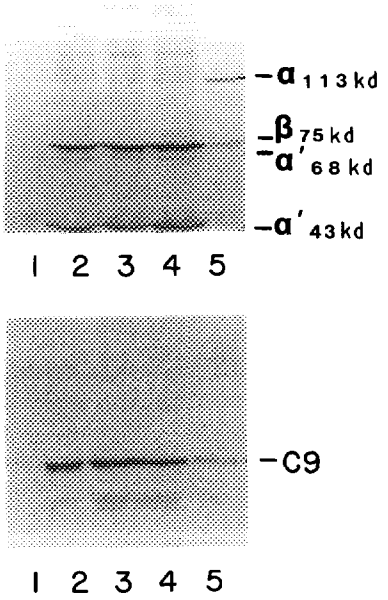
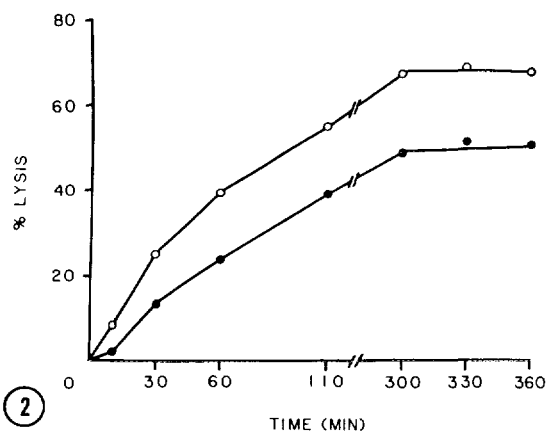


Fig. 1. Uptake of C3 activation fragments and C9 on antibody sensitized human erythrocytes. Human erythrocytes (lane 1), human erythrocytes treated with anti-HSA (lane 2), human E-LPS-Ab (lane 3), or human E-LPS-Ab treated with anti-HSA (lane 4) were incubated with human complement and washed. Solubilized cells and diluted human serum (lane 5) were electrophoresed and transferred to nitrocellulose sheets. Cell associated C3 (upper panel) and C9 (lower panel) were detected with appropriate antisera as described in Materials and Methods.

components with the cell surface. During incubation with complement, both C3 activation fragments and C9 bound to erythrocytes sensitized with anti-HSA even though hemolysis did not occur (Fig. 1, lane 2). E-LPS-Ab, which were lysed nearly 60% by this dilution of human complement, demonstrated immunoblot analysis patterns of C3 and C9 binding that were very similar to that of the previous cells (Fig. 1, lane 3). Although anti-HSA, when bound to E-LPS-Ab, inhibited lysis by more than 60%, the quantity of complement components binding to the cells did not decrease (Fig. 1, lane 4). Analysis with a Zeineh Soft Laser Scanning Desitometer (Biomed Instruments, Inc., Fullerton, CA) of the immunoblots shown in Figure 1 indicated no appreciable differences in amounts of C3 or of C9 bound to E-LPS-Ab compared to the amounts bound to E-LPS-Ab that also have been treated with antibodies to albumin. The latter bound 1.3 times as much C3 as E-LPS-Ab without anti-albumin and 1.08 times as much C9. These figures remain very similar if the total amounts of C3 or C9 bound were compared or if corresponding major bands were compared. The pattern of C3 fragments on the immunoblot indicates that cell bound C3 is predominately in the iC3b state (11) on all three populations of antibody sensitized erythrocytes. Upon activation, C3a is cleaved

from native C3 (consisting of a 113 kd α-chain and a 75 kd β-chain) to form C3b which is able to bind cell surface constituents. C3b is inactivated and converted to iC3b by cleavage of the α-chain forming 68 kd and 43 kd α fragments which remain associated with the β-chain until further breakdown occurs (11). However, the association of C9 indicates that active C3b persisted long enough on these cells to allow activation of the complete complement cascade. These findings suggest that cell-bound anti-HSA inhibits the ability of C9 to lyse erythrocytes without blocking binding of C9 to the cells and without aborting complement activation at an earlier step in the cascade.

In an attempt to confirm that cell-bound anti-HSA inhibits complement lysis by influencing the membrane attack complex phase of the cascade, the effect of these antibodies on reactive lysis was examined. As shown in Fig. 2, anti-HSA inhibited both the extent and the kinetics of lysis. Reactive lysis is an artificial system, utilizing preformed C5b6 plus C7, C8 and C9, and thus, bypasses the requirement for antibody and earlier acting complement components. The ability of anti-albumin antibodies to inhibit reactive lysis argues against the idea that albumin-antibody complexes were shed from cells and activated complement in the fluid phase, thus leading to decreased lytic potential.



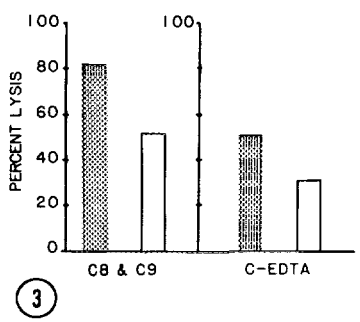


Fig. 2. Kinetics of reactive lysis of untreated human erythrocytes or of human erythrocytes bearing antibodies to HSA. Human erythrocytes to which no antibodies had been added (open circles) or human erythrocytes bearing antibodies to HSA (closed circles) were treated with purified C5b6, C7, and C-EDTA as a source of C8 and C9. Aliquots were withdrawn at various times after the addition of C-EDTA and the percent of cells hemolyzed was determined for each time point.

Fig. 3. Inhibition by cell-bound anti-HSA of reactive lysis: Comparison of purified C8 and C9 to C-EDTA as a source of C8 and C9. The extent of reactive lysis of human erythrocytes which had not been treated with antibody (shaded bars) and of human erythrocytes previously treated with anti-HSA (open bars) were compared using either purified C8 and C9 (left panel) or C-EDTA (right panel) as a source of C8 and C9.

We considered the possibility that the anti-HSA antiserum might be reacting with something in the serum, such as serum albumin, that was used as a source of C8 and C9 to somehow restrict hemolysis. However, cell bound antibodies to HSA inhibited reactive lysis to a similar extent when either C-EDTA was used as a source of C8 and C9 or when all purified components were used (Fig. 3).

The blocking effect of anti-HSA antibody appears to be somewhat akin to that of blocking antibodies found in human sera that inhibit complement-mediated killing of bacteria. The surfaces of erythrocytes and their ability to respond metabolically to complement attack are, of course, much less complex than these properties of bacteria. However, anti-HSA antibodies seem to not only activate complement but also to dictate the outcome of the complement attack as has been described with both bactericidal and blocking antibodies bound to bacteria. Future experiments are required before the mechanism of how anti-HSA antibodies accomplish this is known. Perhaps HSA is complexed to a significant red blood cell surface constituent and the addition of anti-HSA antibody either directs the location of the membrane attack complex, as has been suggested regarding the interaction of blocking antibodies with bacteria, or affects the cell in such a way as to inhibit MAC insertion. Evidence suggests that albumin affects the cytoskeleton of neutrophils because added albumin inhibits aggregation of stimulated neutrophils, apparently by suppressing actin polymerization (12). Liu et al have reported that the membrane attack complex interacts with cytoskeletal components of erythrocytes (13). Therefore, if albumin affects the cytoskeleton of erythrocytes as well as of neutrophils, perhaps the additional effect of antibodies bound to albumin is interference with cytoskeletal-MAC interactions.

Earlier, we reported that erythrocytes are resistant to complement-mediated hemolysis resulting from activation of the classical pathway if the erythrocytes and complement are from the same species (4), this phenomenon is referred to as homologous restriction. Later we showed that unlysed homologous erythrocytes bound C9 and that the cell-bound C9 caused the cells to become resistant to lysis by even heterologous complement (14). Therefore, C9 bound to homologous cells during complement activation and cell-bound antibodies to albumin exert the same effect on erythrocytes. It will be interesting to determine if the mechanisms of protection by these two proteins are similar. However, the ability of anti-albumin antibodies to render the cells resistant to complement lysis does not appear to be directly related to homologous restriction, as cells bearing these antibodies are not lysed by either homologous or heterologous complement.

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REFERENCES

1. Joiner, K.A., Scales, R., Warren, K.A., Frank, M.M., and Rice, R. (1985) J. Clin. Invest. 76,1765-1772.
2. Houle, J.J., and Hoffmann, E.M. (1993) Biochem. Biophys. Res. Comm. 194,1161-1166.
3. Garratty, G. (1991) Gerontology 37,68-94.
4. Houle, J.J., and Hoffmann, E.M. (1984) J.Immunol. 133,1444-1452.
5. Yamamoto, K., and Gerwurz, H. (1978)) J.Immunol. 120,2008-2015.
6. Biesecker, G., and Müller-Eberhard, H.J. (1980). J.Immunol. 124,1291-1296.
7. Houle, J.J. and Hoffmann, E.M. (1987) Vet.Immunol. Immunopath. 16,259-270.
8. Laemmli, U.K. (1970) Nature 227,680-685.
9. Towbin, H.T., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76,4350-4354.
10. Houle, J.J., Hoffmann, E.M., and Esser, A.F. (1988) Blood 71,280-286.
11. Becherer, J.D. Alsenz, J., and Lambris, J.D. (1990) Curr. Topics Microbiol. Immunol. 153,45-72.
12. Colli, S., Marinovich, M., Stragliotto, E., Galli, C.L., and Tremoli, E. (1989) Biochem. Pharmacol. 38,3909-3912.
13. Liu, Z.-Y., Sanders, M.E., and Hu, V.H. (1989) J. Immunol. 142,2370-2376.
14. Houle, J.J., Hoffmann, E.M., and Esser, A.F. (1988) Blood 71,287-292.