Restriction of Cell Lysis by Homologous Complement: II. Protection of Erythrocytes Against Lysis by Newly Activated Complement

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Our previous work revealed that homologous complement (C) was ineffective in lysing antibody-sensitized erythrocytes (EA) even at high concentrations. It was also shown that activation of complement on homologous EA resulted in the binding of C9 and the formation of EA bearing complement proteins C1 through C9 (EAC1-9), yet few hemolytic sites were formed. Instead, as shown here, the formation of homologous EAC1-9 caused the cells to become resistant to lysis even by heterologous complement during a second incubation. In contrast, when homologous EAC1-8 were produced by incubating EA with C9-depleted serum, such intermediates were not protected against lysis by heterologous complement during a second incubation. Furthermore, homologous C9 on EAC1-9 was able to reduce the hemolytic efficiency of heterologous complement without blocking C activation and the formation of new C5b-9 complexes. Protection was not modified when homologous EAC1-9 were produced in one step, by incubation of EA with serum, or sequentially by adding C9 to EAC1-8. The minimum number of 9-sites required to confer a protective effect on EAC1-9 was less than 200 per cell. Thus, in addition to its known effect in heterologous cell killing, homologous C9 is capable of protecting homologous cells against inadvertent complement lysis.

In a preceding study, Houle and Hoffmann compared the abilities of various sera to lyse erythrocytes (E) from six different species and found that complement from all six species lysed heterologous antibody-sensitized erythrocytes (EA) much more effectively than homologous EA. They also observed that homologous serum could actually inhibit lysis by heterologous serum. Furthermore, we reported that fresh, antibody-sensitized human erythrocytes (EhUA) were only partially lysed by incubation with even large amounts of undiluted human serum. Moreover, during the course of these studies we noticed that, once formed, homologous EA bearing complement proteins C1 through C9 (EAC1-9) were also more resistant to lysis by heterologous complement during a second incubation. This suggested the possibility that the two phenomena, i.e., restriction of lysis and protection of homologous EAC1-9 against heterologous lysis, were caused by the same mechanism or closely related ones. Further examination of the protection process revealed that activation of the complement cascade was required and that EAC1-9 remained protected against lysis by heterologous complement even after several washing steps.

Analogous reactions may occur in vivo in patients with chronic cold agglutinin disease, which is characterized by autoantibodies to erythrocytes. These patients' erythrocytes are more resistant than are normal human erythrocytes to complement-mediated hemolysis both in vivo and in vitro, and the resistance appears to be associated with an accumulation of complement proteins on such erythrocytes. Because of the medical implications of our earlier findings and the possibility that elucidation of the “protection” mechanism might shed light on the “restriction” mechanism, we decided to investigate the mechanism(s) that protect(s) homologous EAC1-9 against immune lysis in more detail. Our results indicate that binding of homologous C9, in association with complement components C5b-8, to homologous erythrocytes is required for protection. Parts of this study were presented earlier in preliminary form.

MATERIALS AND METHODS

Sources for erythrocytes, antigens, antibodies, and methods for erythrocyte sensitization were exactly as described in the preceding paper. Likewise, preparation of complement component-depleted sera and isolation and radiiodination of complement proteins followed procedures detailed earlier.

Binding studies. EwA were incubated for 60 minutes at 37°C with appropriately diluted human serum or with C8- or C9-depleted sera that had been reconstituted to physiological levels with 125I-C8 or 125I-C9, respectively. The cells were pelleted and washed three times with veronal-buffered saline containing 0.15 mmol/L CaCl2, 0.5 mmol/L MgCl2, and 1% gelatin (GVB), and the associated radioactivity was determined. Corrections for nonspecific binding were made by subtracting counts associated with cell pellets that had been incubated with radiiodinated proteins in the absence of serum.

Protection assays. EA (5 x 10⁷ cells/mL) were incubated for 30 minutes at 37°C with equal volumes of either whole serum or various depleted sera. The mixtures were centrifuged, and the amount of lysis was determined. The surviving cells were washed two times, restandardized to 5 x 10⁷ cells/mL, and used in a second incubation step to determine whether they were protected against lysis by either homologous or heterologous sera.

Kinetic lysis assays. The rate of hemolysis in isotonic ammonium chloride was determined by light scattering. Appropriately diluted cells were placed into a 3-mL cuvette in a Hewlett-Packard 8450A spectrophotometer, and transmittance was monitored at 610 nm. The temperature was maintained at 37.0°C, and the suspension was continuously stirred. Lysis caused a decrease in absorbance.

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(forward light scattering), and the time required to reach a 50% absorbance change after addition of the lytic agent was used to determine the lysis halftime (t_{50}) as described.  

RESULTS

**Immune lysis of EAC1-8 and EAC1-9.** To test which cell intermediates would be protected against lysis by heterologous complement, we measured lysis of EwA, EwAC1-8, and EwAC1-9 during a second incubation with guinea pig complement. The intermediates were prepared by incubating EwA with either buffer (GVB), C8-depleted serum (diluted 1:10), C9-depleted serum (1:10), or with C8- or C9-depleted sera (1:10) that had been restored with physiological amounts of C8 (6 μg/mL) or C9 (6 μg/mL), respectively. This first incubation resulted in 22% lysis by C9-depleted serum and in 19% lysis by C8-depleted serum that had been restored separately with C8 or C9, respectively; and there was no significant lysis in the other incubation mixtures. The surviving cells—EwA, EwAC1-7, EwAC1-8, and EwAC1-9, respectively—in each sample were washed, restandardized to the original concentrations, and exposed to guinea pig complement (1:40) for the second incubation. Compared with EwA or EwAC1-8, EwAC1-9 were resistant to lysis by guinea pig complement (Fig 1, left panel), which suggests that C9 is somehow involved in protecting cells against subsequent immune lysis. Analogous results were obtained when EwAC1-7 were compared with EwAC1-9 (Fig 1, right panel); again only EwAC1-9 were protected. These results demonstrate the necessity for C9 in producing protected EwAC1-9. They also show that C9 must be bound to elicit this effect and that it is not effective when merely present during the first incubation because cells incubated with C8-depleted serum that contains C9 are sensitive to lysis by guinea pig serum. Furthermore, incubating EwA with C9 in the absence of C9-depleted serum has no effect on the subsequent lysis of the cells by guinea pig complement (Fig 1, right panel).

**Protection against lysis is not absolute.** On the basis of the results of the previous experiments one could propose that all available binding sites for new membrane attack complexes (MACs) were occupied on the target cells after the first incubation with homologous serum. If this were true, then even high concentrations of heterologous complement should not be able to lyse all protected cells. However, this was not the case. EwA serving as controls and EwAC1-9 produced by incubation with undiluted human serum were incubated with serial dilutions of human (Fig 2, right panel) or guinea pig serum (Fig 2, left panel). High concentrations of guinea pig complement (twofold diluted) lysed 100% of the protected EwAC1-9, thereby suggesting that at least some sites remained for guinea pig MAC deposition on the target membranes. However, even undiluted human serum could not overcome protection as shown by the fact that EwAC1-9 were lysed less than were control EwA.

**Heterologous C9 binding is not blocked on protected EAC1-9.** It is conceivable that the observed “protection” phenomenon resulted from blockage of C5b-9 deposition during the second incubation. To test for this possibility we determined the rate and extent of human 125I-C9 binding to protected bovine EA bearing complement proteins C1 through C9 (EwAC1n-9). This was done by incubating 2.6 × 10⁶ EwA or EwAC1n-9 in 1.3 mL total volume with 0.39 mL human serum containing 3.9 μg 125I-C9 at 37°C and measuring both hemolysis and C9 binding at different times. As shown in Fig 3, control EwA were lysed completely within 15 minutes and after 60 minutes had taken up ~8 × 10⁵ 125I-C9 molecules per cell, whereas only ~56% of the protected EwAC1n-9 were lysed after the same length of incubation, although they bound about 2.5 times as much 125I-C9 (~21 × 10⁵ radiolabeled molecules/cell) at the end point. The newly added heterologous 125I-C9 did not just “fill in” vacant sites on homologous MACs on EwAC1n-9 because the addition of human 125I-C9 contained in human serum that had been inactivated by the addition of 10 mmol/L EDTA did not result in a significant uptake of C9 by these cells (Fig 3, dashed line). In addition, no impairment of C8 binding was observed when human 131I-C8 was used to measure C5b-9 formation on such protected bovine cells, which indicated that new MACs are formed during the second incubation. These results also show that the C9:C8 ratio (~4.8) in these newly deposited heterologous MACs is more than twice as great on protected cells (EwAC1n-9) as on control cells (EwA) where C9:C8 approximates 2.3. Furthermore, no loss of C9 from protected EAC1-9 was observed (using 131I-C9 as a tracer) during the second incubation with heterologous serum (data not shown). These results provide further evidence against trivial explanations such as release of C9 from protected cells to allow uptake of heterologous C9 on nonlytic 8-sites, filling in of incomplete sites, or that protection results from blockage of C5b-9 formation during the second incubation.

**Protection is independent of the sequence of EAC1-9 formation.** Protected EwAC1n-9 were prepared by two different protocols. EwA were incubated either with C9-
depleted serum (diluted 1:10) that had been reconstituted with physiological concentrations of human C9 to form EhUAChU1-9 or with C9-depleted serum to form EhAChU1-8 intermediates; the latter cells were then washed three times and incubated with human C9 (6 μg/mL) to form EhUAChU1-9. After restandardization to the original concentration, all of the cells were then incubated with guinea pig serum, and hemolysis was measured after 60 minutes' incubation at 37°C, and as demonstrated in Fig 4, both cell preparations were protected equally well.

Protection mediated by C9 is concentration dependent. The effect of decreasing amounts of C9 added to a fixed volume of C9-depleted serum to render EhA resistant to subsequent lysis by guinea pig serum was determined. The protection assay was performed as described in Materials and Methods. EhUAChU1-9 were produced by incubating EhA with C9-depleted human serum (diluted 1:20) that had been restored with decreasing concentrations of C9 (150 to 0.06 μg/mL). After the cells were washed and restandardized, they were incubated a second time with guinea pig serum (diluted 1:90). In Fig 5, the average number of hemolytic sites per cell (Z = -ln of fraction of unlysed cells) formed by incubation with guinea pig complement is plotted vs the concentration of C9 used in the first incubation. There is a sharp rise from about 0.7 to 1.7 hemolytic sites when less than 0.6 μg C9/mL was used in the first incubation, which indicated that C9 must be present in about equal concentrations to the other terminal components to be effective. Furthermore, supraphysiological concentrations of C9 that foster polymerized C9 (poly C9) formation had no effect on the number of hemolytic sites that were produced.

We also attempted to measure the minimum amount of C9 that must be bound to homologous cells to achieve protection. For these experiments 125I-C9 was added to human serum as a tracer or was used to reconstitute C9-depleted serum, and both sera were diluted 80-fold with GVB. EhA were then incubated for 60 minutes with these sera, and as shown in Table 1, very little lysis occurred during this first incubation. Nevertheless, protected EhUAChU1-9 were formed during this step because these cells were resistant to lysis by guinea pig serum despite the fact that the amount of C9 bound during the first incubation was less than 200 molecules per cell, which is the detection limit of our current binding assay.

Protected cells are susceptible to ammonia lysis. RBCs are protected against colloid-osmotic lysis because of the low cation permeability of their plasma membranes. However, these membranes are highly permeable to uncharged ammonia, and when cells are placed in isotonic NH4Cl, NH3 penetrates into the cells where a new equilibrium is established: NH3 + H2O → NH4+ + OH-, and the hydroxyl ion is exchanged with the external anion (Cl-). The net result is the penetration of NH4Cl, a process that leads to swelling and finally to osmotic hemolysis. To test whether protected EAC1-9 cells were generally more resistant to hemolysis or specifically to complement-mediated lysis, we used the aforementioned lysis procedure and compared protected EAC1-9 and unprotected normal erythrocytes and EA for their susceptibility to ammonia lysis. When the three different cell
formation. E,A

The average number of hemolytic sites per EAC1-9 demonstrated that activation of homologous EA generates cell-bound C5b-9 complexes, yet few hemolytic sites are formed. We are now able to show that homologous C9 that is a part of such C5b-9 complexes causes homologous target cells to resist lysis even by heterologous complement. When homologous serum was depleted of C9 and used to produce EAC1-8, these intermediate cells were not protected against subsequent lysis by heterologous serum. The mere presence of C9 was not sufficient to produce protected cells because EAC1-7 intermediate cells that had been produced by incubation with C8-depleted serum and, therefore, in the presence of C9 were not protected. As expected, C8- and C9-depleted sera when restored with the missing protein were capable of producing protected EAC1-9, and more importantly, unprotected EAC1-8 could be converted to protected EAC1-9 by incubation with homologous C9.

Investigations into possible mechanisms through which bound C9 was able to render homologous cells resistant to lysis by heterologous complement eliminated several trivial explanations. For example, one could postulate that heterologous complement did not lyse EAC1-9 because no further uptake of heterologous C5b-9 was possible due to occupation of all binding sites. This possibility could be eliminated because direct binding studies revealed that heterologous C9 was taken up during the second incubation. Importantly, the amount of C9 that bound was actually 2.5 to 3 times higher than would normally bind to unprotected cells. The opposite possibility that heterologous C9 was filling in vacant sites on homologous MACs and, therefore, was not lytic could also be eliminated because no significant uptake of C9 occurred unless heterologous complement activation occurred that led to formation of new MACs. Protected cells also bound additional C8, which indicated that heterologous MACs indeed are formed during the second incubation. The ratio of heterologous C9 to C8 was higher on protected cells than on control cells. Whether this higher ratio relates to our earlier finding2 that formation of poly(C9) is enhanced on homologous EA that resist lysis is not clear and awaits further study.

These results also indicate that bound homologous C9 does not start a feedback reaction that attenuates formation of heterologous C3/C5 convertase complexes; rather, it is the ensuing interaction between the newly deposited heterologous C5b-9 complexes and the target membrane that determines the less efficient lysis. The fact that very high concentrations of heterologous complement could completely lyse EAC1-9 also speaks against the possibility that uptake of C5b-9 was blocked during the second incubation.

A third possible mechanism could have been exchange of bound homologous C9 for added heterologous C9 without the formation of new hemolytic sites. When the fate of bound, homologous 1251-C9 was followed, however, no release could be detected during the second incubation. Furthermore, because uptake of heterologous C8 could also be demonstrated, it is apparent that new, heterologous C5b-9 complexes were formed on protected target cells. In summary then, these data clearly demonstrated that new C5b-9 sites were formed on protected target cells but that such complexes were hemolytically ineffective and, thus, further studies into the actual mechanism(s) that lead to protection were required.

To formulate any kind of working hypothesis it was important to determine the amount of C9 required to confer protection. Titration of C9 required to reconstitute C9-depleted serum to produce protection indicated that C9 must be present in about equal molar concentrations to the other
terminal components. This result argues against the possibility that poly(C9) formation was required for protection because Bhakdi and Tranum-Jensen have recently reported that the concentration of C9 in serum must be artificially increased or large amounts of serum must be used before poly(C9) formation becomes significant on heterologous erythrocytes. Obviously, there must also be a minimum number of homologous C5b-9 complexes for protection to occur. We have attempted to measure this number and found that it is below the present limit (~200 C9 molecules/cell) of our assay system, and further studies are underway to determine this threshold more precisely. Nevertheless, the low number already suggests that protection is probably not achieved by direct molecular interactions between homologous C9 within deposited C5b-9 complexes and heterologous C5b-9 complexes because as many as 22,000 heterologous C9 molecules per protected cell are taken up during the second incubation.

At this time two possible mechanisms for protection can be suggested. It is conceivable that the slow deposition of homologous C5b-9 during the first incubation affects membrane properties locally or globally by allowing influx of Ca2+, which may trigger a series of as yet undefined reactions that make red cells more resistant in general. For example, insertion of C9 into the target membrane may be decreased on protected cells analogously to the difference for C9 insertion on sheep and human erythrocytes reported by Hur and Shin. Alternatively, homologous C5b-9 might interact specifically with an as yet undefined membrane constituent on homologous cells—which possibly could be an earlier acting homologous complement protein—and thereby could trigger these reactions. However, the cells do not become more resistant to lysis in general because protected and unprotected cells are equally susceptible to ammonia lysis and, thus, protection appears to be specific for complement-induced lysis.

Although it is clear that C9 is required for protection of homologous EA, it is obviously not acting alone in this capacity. C9 must interact with target membrane constituents, either directly or indirectly, that allow it to distinguish between homologous and heterologous erythrocytes. Also, because attachment of C9 to cells requires formation of the MAC, involvement of earlier-acting proteins cannot yet be eliminated. Two proteins have recently been extracted from human membranes that restrict C8 and/or C9 activity. One of these proteins, C8-binding protein (C8-bp), binds to and inhibits the activity of human C8 but not rabbit C8. Although C8-bp may play an important role in the restriction of homologous cell lysis, it is difficult to envision how it could function in protection of homologous erythrocytes against lysis by newly activated heterologous complement because large numbers of new heterologous MACs are formed and the protein is thought to be specific for homologous C8. The second inhibitory protein, called human restriction protein, is thought to block C9 channel and poly(C9) formation but is also species specific. Serum high-density lipoproteins (HDL) are also known to inhibit C9 hemolytic activity. However, it was reported that HDL was only minimally effective if added before C9, and because purified C9 can convert unprotected EAC1-8 to protected EAC1-9 in the absence of HDL, it seems unlikely that these proteins are required for protection. Thus, if additional serum proteins are required for protection, they must bind to cells during EAC1-8 formation.

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Table 1. Nonlytic Amounts of C9 Are Capable of Protecting Homologous EAC1-9

<table>
<thead>
<tr>
<th>Target Addition</th>
<th>First Incubation* Lysis (%)</th>
<th>C9 Bound per Cell</th>
<th>Second Incubation† Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EλA GV9</td>
<td>&lt;3</td>
<td>0</td>
<td>EλA GPS 80</td>
</tr>
<tr>
<td>EλA C9-depleted serum</td>
<td>&lt;3</td>
<td>0</td>
<td>EλA C9-depleted serum 1:90 GPS 57</td>
</tr>
<tr>
<td>EλA C9 + C9-depleted serum 1:90</td>
<td>&lt;3</td>
<td>&lt;200</td>
<td>EλA C9-depleted serum 1:90 GPS 39</td>
</tr>
<tr>
<td>EλA Human serum (1:90)</td>
<td>&lt;3</td>
<td>&lt;200</td>
<td>EλA C9-depleted serum 1:90 GPS 39</td>
</tr>
</tbody>
</table>

Abbreviations: GPS, guinea pig serum (1:90).

*EλA (5 x 10⁷ cells/mL) were incubated with equal volumes of the reagents listed, and the percentage of cells lysed was determined. The surviving cells were washed and resuspended to the original concentration.

†Surviving cells from the first incubation were incubated with guinea pig complement.

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Fig 6. Ammonia lysis of normal and protected erythrocytes. Human E (--), EA (—), and EAC1-9 (---) at equal concentrations were suspended in isotonic ammonium chloride at time 0, and lysis was measured by monitoring forward light scattering at 610 nm.
In summary, as discussed earlier by us\textsuperscript{12} and others,\textsuperscript{10,13} restriction of complement lysis on homologous cells is a complex process that involves multiple steps in the complement cascade. Described here is an interesting aspect of restriction, that of protection of homologous cells by C9. It appears that uptake of C9 on homologous EAC1-8 made such cells even more resistant to complement activation occurring after the initial C9 binding and MAC formation.

Elucidating reactions and mechanisms involved in protection may help to clear molecular details of the general phenomenon of homologous restriction.

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Restriction of cell lysis by homologous complement: II. Protection of erythrocytes against lysis by newly activated complement

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