Effect of C3b Inactivator on Monocyte-bound C3-coated Human Erythrocytes

By Alan D. Schreiber and Patricia B. McDermott

As a model of IgM-induced hemolytic anemia in man, human erythrocytes were sensitized with IgM antibody and coated with complement components, including C3 and C4, using human serum as a source of complement. These coated red cells were then interacted with monolayers of human mononuclear phagocytic cells (monocytes). Complement-coated red cells so bound could be displaced from their monocyte attachment site in a dose- and time-dependent manner by serum factors, including C3b inactivator (C3bINA). These factors were more efficient in inactivating red cell-bound complement components prior to interaction of the coated cells with monocytes. With large amounts of complement per erythrocyte, measured as membrane-bound C3, the ability of the serum inactivating factor(s) to remove the complement-coated red cells from the monocyte surface was compromised and persistently bound red cells were progressively phagocytosed. These studies implicate C3bINA in the displacement of complement-coated erythrocytes, formed from the interaction of IgM antibody and serum complement, from the hepatic macrophage in IgM-induced immune hemolysis. They suggest that both the concentration of complement components, especially on the erythrocyte surface, and the level of C3bINA and perhaps other inactivators may be important features regulating hemolysis in this disorder.

THE MACROPHAGE COMPLEMENT RECEPTOR is important in host defense and, in part, is responsible for the increased clearance of complement-coated cells in patients with autoimmune hemolytic anemia and autoimmune thrombocytopenia. Erythrocyte destruction in patients with IgM-induced immune hemolysis, e.g., cold hemagglutinin disease, results from activation of the classic complement pathway and the deposition of a fragment of the third complement component, C3b, on the erythrocyte surface. These C3-coated cells are sequestered by hepatic macrophages of the reticuloendothelial system. However, a portion of these cells are released from their macrophage attachment site, whereupon they circulate normally.

To investigate these interactions in vitro we employed a quantitative model using human components: erythrocytes, complement, and peripheral blood mononuclear phagocytic cells, cells closely related to tissue macrophages. Our results implicate the plasma protein C3b inactivator as one important molecule modulating the release of C3-coated cells from the macrophage surface.
Isotonic veronal-buffered saline pH 7.4 containing 0.1% gelatin, 0.00015 M CaCl₂, and 0.0005 M MgCl₂ (VBS) and veronal-buffered saline containing 0.1% gelatin and 0.01 M ethylenedinitrilotetraacetic acid (EDTA buffer) were prepared as previously described. Antiserum monospecific for the plasma protein β1H was a gift from Dr. Ulf Nilsson, University of Pennsylvania School of Dental Medicine. Hanks’ balanced salt solution (HBSS) (Grand Island Biological, Grand Island, N.Y.), rabbit monospecific antiserum to human IgM, IgG, and C3 (Behring Diagnostic, Somerville, N.J.), fresh-frozen guinea pig serum (Rockland, Gilbertsville, Pa.) and C3 (Cordis Laboratories, Miami, Fla.) were obtained as indicated. Partially purified guinea pig C2, human C1, and cells coated with hemolytic antibody and the fourth component of complement (EAC4) were prepared by a modification of established methods.

**C3b inactivator (C3bINA).** Two sources of C3bINA were used in these studies, autologous human serum heat inactivated (56°C for 45 min) and partially purified C3bINA obtained from Cordis Laboratories. This partially purified C3bINA was further characterized. It contained no antigenically detectable β1H, C3, or C3 split products by Ouchterlony and immunoelectrophoretic analyses. Immunoelectrophoresis developed with rabbit anti-human antiserum showed two bands, a γ globulin that was identified as IgG and a β globulin corresponding to C3bINA.

The partially purified C3bINA was also subjected to alkaline disc gel electrophoretic analysis and stained with Coomassie blue. Again two major protein bands were detected. The cathodal band corresponded to IgG and the anodal band to C3bINA when unstained replicate gels were sliced and eluted (Fig. 1).

C3bINA activity was assayed in this and other experiments by inhibition of immune adherence using IgM-coated sheep erythrocytes sensitized with the sequential addition of the purified components C1, C4, C2, and C3 so as to carry five hemolytic C3 sites per cell. These C3b-coated erythrocytes (2 x 10⁶) were immune adherence positive. They were incubated at 37°C for 120 min with serial twofold dilutions of C3bINA source in VBS containing 5% dextrose, resuspended, and then 1 x 10⁶ cells added to 2 x 10⁶ washed human erythrocytes (possessing the immune adherence receptor) in microtiter plates for 45 min at 37°C. The reciprocal of the dilution of C3bINA source producing 50% inhibition of immune adherence was designated as a relative C3bINA concentration of unity.

**Preparation of human erythrocytes sensitized with IgM and C3 (ElgMC3).** Rabbit IgM antibody to human erythrocytes was prepared as previously described and isolated by Sephadex G-200 chromatography. The IgM peak was pooled so as contain no detectable IgG by Ouchterlony analysis and stored at 4°C. Monospecific IgG anti-human C3 was also isolated as previously described. This anti-C3 did not recognize IgG by immunoelectrophoretic and Ouchterlony analyses and did not agglutinate IgG-coated RBC. This anti-C3 recognized the C3 degradation products C3b and C3d but preferentially recognized C3b as determined by immunoelectrophoretic analysis and by its ability, when radio labeled, to adsorb to C3b-coated erythrocytes as opposed to C3d-coated (C3bINA treated, immune adherence negative) erythrocytes.
Washed human erythrocytes were suspended to $1.1 \times 10^8$ RBC/ml and sensitized with an equal volume of IgM antierthrocyte antibody in EDTA buffer for 30 min at 37°C, washed once with EDTA buffer and twice with VBS. These RBC (2.5 $\times 10^7$) were incubated with fresh autologous serum in VBS as a source of C3 at 37°C for 15 min, washed once with VBS and twice with HBSS, and resuspended to $5 \times 10^7$/ml with HBSS. After this incubation period, these complement-coated RBC, operationally termed ElgMC3, carried C3b, since they were immune adherence positive. Under the physiologic conditions in which these cells were prepared they undoubtedly contained other complement components, including C4b and small amounts of C3d. The number of IgM and C3 molecules per RBC was determined by C1 fixation and transfer. In experiments utilizing ElgMC3 both control erythrocytes sensitized with IgM alone and erythrocytes exposed to serum in the absence of IgM antibody were employed. Erythrocytes sensitized with subagglutinating concentrations of IgM antibody were not agglutinated by anti-IgG antisera.

**Mononuclear cell C3 receptor assay.** Mononuclear cell monolayers were prepared as previously described using human peripheral blood mononuclear cells obtained from Ficoll-diatrizoate density gradient centrifugation. Monolayers contained greater than 97% mononuclear cells, of which approximately 80% were peroxidase and esterase positive. Approximately 90% of cells morphologically appeared to be monocytes when stained with Wright-Giemsa and examined by light microscopy, and 75% of the cells ingested latex particles.

Mononuclear cell binding of ElgMC3 was achieved by applying 1 ml of $5 \times 10^7$ ElgMC3 to each monolayer (approximately $1 \times 10^5$ cells) and sedimenting in microtiter plate centrifuge holders (Cooke Engineering, Alexandria, Va.) at room temperature for 5 min at 70 g. The monolayers were then incubated for 10 min at room temperature, washed five times with HBSS, air dried, and stained with Wright-Giemsa. One hundred consecutive mononuclear cells were examined, and both the average number of RBC bound per mononuclear cell and the percentage of mononuclear cells binding at least three RBC were determined. With increasing concentrations of C3 per RBC, mononuclear cell binding of ElgMC3 progressively increased (Fig. 2). No mononuclear cell binding of control ElgM or of control E exposed to autologous serum in the absence of antibody was observed. Percentage inhibition by C3bINA of ElgMC3 mononuclear cell binding was determined by comparison to buffer treated controls:

$$\text{Percentage inhibition or inactivation} = \left( \frac{\text{Monocyte-associated ElgMC3 following incubation with C3bINA}}{\text{Monocyte-associated ElgMC3 following incubation with buffer}} \right) \times 100.$$  

**Phagocytosis of ElgMC3** by mononuclear cells was assessed by incubating washed monolayers containing monocyte-bound ElgMC3 with 1 ml HBSS at 37°C in a humid atmosphere of 5% CO₂ in air. The monolayers were washed with HBSS, and 1 ml of 0.15 M NaCl was added to each plate followed by 3 ml distilled H₂O for 30 sec (to remove uningested but bound RBC) and 1 ml of

![Fig. 2. Effect of C3 per erythrocyte on ElgMC3 binding by human monocytes.](image)
Fig. 3. Removal of monocyte (M)-bound ElgMC3 by C3bINA. Percentage ElgMC3 removed from monocyte surface determined (A) by observing RBC/M or (B) percentage of M binding ≥ 3RBC before and after exposure to C3bINA. •, ElgMC3 exposed to C3bINA before incubation with monocytes; ■, ElgMC3 bound to monocytes before exposure to C3bINA. Brackets, mean ± SEM as evaluated by Student's t test for unpaired samples.

3.6”, NaCl. Monolayers were then air dried and stained with Wright-Giemsa, and the percentage of mononuclear cells containing phagocytosed RBC was determined by light microscopy.

RESULTS

To examine the role of the C3bINA system in regulating the interaction between complement-coated erythrocytes and the monocyte complement receptor, ElgMC3 were either preincubated with C3bINA and washed prior to exposure to mononuclear cell monolayers or, in parallel, C3bINA was incubated with ElgMC3 that had first been bound to the mononuclear cell surface.

When ElgMC3 were first bound to mononuclear cells and then exposed to partially purified C3bINA at 37°C for 120 min, they were progressively removed from their mononuclear cell attachment site by increasing concentrations of C3bINA (Fig. 3). High concentrations of C3bINA were required to displace more than 80% of ElgMC3. However, when C3bINA was preincubated with $5 \times 10^7$ ElgMC3 before exposure to the mononuclear cell monolayers, less C3bINA was required to prevent the attachment of these erythrocytes to the mononuclear cells than was necessary to cause their removal from the mononuclear cell surface (Fig. 3). On the other hand, when C3bINA in heat-inactivated serum was studied, it appeared equally effective in removing mononuclear cell-bound ElgMC3 and in preventing ElgMC3 preincubated with this C3bINA from binding to the mononuclear cell surface (Fig. 4). ElgMC3 exposed
to C3bINA either before or after mononuclear cell attachment were immune adherence negative, indicating inactivation of erythrocyte bound C3b.

The time course of inactivation of ElgMC3 by C3bINA in serum is shown in Table 1: 66% inhibition of ElgMC3 binding was observed within 60 min of incubation with C3bINA and 72% inhibition within 120 min. However, by 60 min C3bINA in serum was capable of displacing only 32% of ElgMC3 from their mononuclear cell attachment site.

In order to further examine the role of C3bINA in plasma on mononuclear cell bound ElgMC3 we studied the plasma obtained from a patient (T.J.) with

**Table 1. Kinetics of Removal of Mononuclear Cell (M)-bound ElgMC3 by C3bINA**

<table>
<thead>
<tr>
<th>Time of Incubation With C3bINA (min)</th>
<th>ElgMC3 Inactivated* (%)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Prior to M Binding (A)</td>
<td>After M Binding (B)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>16 (6, 25)</td>
<td>17 (12, 22)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>66 (65, 67)</td>
<td>32 (30, 33)</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>72 (73, 70)</td>
<td>60 (50, 70)</td>
<td></td>
</tr>
</tbody>
</table>

C3bINA (relative concentration 16) in heat-inactivated serum was incubated at 37°C with mononuclear cell-bound ElgMC3. The time course of ElgMC3 removal (B) was determined and compared to that of ElgMC3 inactivated by C3bINA prior to mononuclear cell association (A). Results were determined by observing the number of RBC/M in comparison to controls in which buffer was substituted for C3bINA. The results of the individual experiments are in parentheses.

*Mean of two experiments.
Table 2. Removal From Mononuclear Cell (M) Surface of Bound ElgMC3 by C3bINA:
Effect of Increasing C3/RBC

<table>
<thead>
<tr>
<th>C3/RBC (molecules)</th>
<th>ElgMC3 Remaining Bound to M Following C3bINA (%)</th>
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<tbody>
<tr>
<td>662</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>1,325</td>
<td>29 (26, 32)</td>
</tr>
<tr>
<td>2,650</td>
<td>26 (20, 32)</td>
</tr>
<tr>
<td>5,300</td>
<td>65 (68, 62)</td>
</tr>
<tr>
<td>10,600</td>
<td>72 (76, 68)</td>
</tr>
</tbody>
</table>

Mean of two experiments. Results were calculated by comparing the monocyte-associated ElgMC3 following incubation with either C3bINA or buffer, as described in Materials and Methods. The results of the individual experiments are in parentheses.

the congenital absence of C3bINA, kindly supplied by Dr. Chester A. Alper (Center for Blood Research, Harvard Medical School, Boston, Mass.). Cell-bound ElgMC3 were incubated as above for 120 min with normal or C3bINA-deficient plasma. While normal plasma (relative concentration 32) displaced a mean of 80% of ElgMC3, the same concentration of C3bINA-deficient plasma displaced only 24% ElgMC3 from the mononuclear cell surface.

We next examined the effect of increasing the number of C3 molecules per erythrocyte on the capacity of C3bINA to remove these mononuclear cell-bound erythrocytes (Table 2). Erythrocytes were sensitized with increasing concentrations of C3 and these cells were bound to mononuclear cells and then exposed to a single concentration of C3bINA in serum. Similar to the results shown in Fig. 2, as the number of C3/RBC increased, the binding of these cells to the mononuclear cell surface increased. At low concentrations of C3/RBC most of these ElgMC3 were displaced from the mononuclear cell surface. As the concentration of C3 per RBC was increased, however, the percentage of ElgMC3 that continued to remain mononuclear cell bound after exposure to C3bINA was increased.

The fate of mononuclear cell-bound ElgMC3 unremoved by C3bINA was also studied. When erythrocytes were sensitized with high concentrations of C3 (5300 C3/RBC) and were bound to the mononuclear cell surface and not removed by C3bINA, an increasing percentage of these cells were phagocytosed over several hours (Table 3). Unphagocytosed ElgMC3 in the absence of C3bINA remained bound to their mononuclear cell attachment site.

DISCUSSION

This study indicates that human C3bINA, both partially purified and in serum, can cause the removal of complement-coated (including C3b) erythro-

Table 3. Phagocytosis of Mononuclear Cell (M)–bound ElgMC3 in Absence of C3bINA

<table>
<thead>
<tr>
<th>Time of M–ElgMC3 Incubation (hr)</th>
<th>Phagocytosis* (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>5 (4, 5)</td>
</tr>
<tr>
<td>1.5</td>
<td>10 (8, 11)</td>
</tr>
<tr>
<td>4</td>
<td>25 (19, 31)</td>
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</table>

Mean of two experiments.

*Percentage of M containing phagocytosed ElgMC3. The results of the individual experiments are in parentheses.
cytes bound to monolayers of monocytes (Figs. 3 and 4). This effect is dependent both upon the concentration of C3bINA and the time of incubation of C3bINA with monocyte-bound E1gMC3 (Table 1). The kinetics of E1gMC3 removal from the surface of human monocytes are similar to those observed in vivo in guinea pig, human, and rabbit models, where E1gMC3 bound to hepatic macrophages are progressively removed and returned to the circulation over 120 min. A similar parallel to events in vivo is the observation that an increase in the number of C3 molecules per erythrocyte results in inefficient E1gMC3 displacement from the mononuclear phagocytic cell surface by C3bINA (Table 2). Thus our results in vitro are in general agreement with those obtained in vivo and indicate that both the concentration of C3 on the erythrocyte surface and the amount of C3bINA available appear important in regulating the fate of E1gMC3 bound to mononuclear phagocytic cells.

If E1gMC3 are not removed from the mononuclear phagocytic cell surface by C3bINA they may be phagocytosed over time (Table 3). This probably explains the fate of hepatic sequestered erythrocytes (E1gMC3), which are not returned to the circulation. Mononuclear phagocytic cells generally ingest E1gMC3 poorly in animal models. However, there is evidence both in vivo and in vitro that macrophage activation considerably facilitates the ingestion of these C3b-coated erythrocytes. Monolayers of mononuclear cells prepared in vitro may behave like the fixed phagocytes of the liver and spleen by efficiently binding E1gMC3 and over time stimulating their phagocytosis. This may reflect activation of fixed monocytes on monolayers.

C3bINA has been previously shown to remove the active C3 fragment (C3c) necessary for immune adherence from the erythrocyte surface. Although serum proteins in addition to C3bINA may be required, the resultant erythrocyte associated C3d is negative in the immune adherence reaction. Human monocytes and macrophages are capable of binding C3d-coated erythrocytes formed with purified components; however, as in our study, when fresh human serum is employed to form E1gMC3, simulating conditions in vivo, C3d-coated erythrocytes formed by C3bINA treatment are poorly bound to monocytes. Whaley and Ruddy have observed that a plasma protein, β1H, facilitates C3bINA activity. One possibility that would explain these observations is that β1H or a similar molecule becomes erythrocyte associated during the formation of the C3b site in whole serum and upon subsequent exposure to C3bINA facilitates C3bINA conversion of C3b to a form of C3d less well recognized by the monocyte or macrophage. In fact there is now evidence to suggest that β1H can bind directly to C3-coated erythrocytes. In our experiments, C3bINA in serum appeared to remove E1gMC3 from the monocyte surface more efficiently than partially purified C3bINA (Figs. 3 and 4). This suggests that another serum protein(s) facilitates E1gMC3 removal from the mononuclear phagocytic cell surface. Such a serum protein(s), which might act in consort with or independently of C3bINA, may be responsible for the capacity of C3bINA-deficient plasma to displace a small number of cell-bound E1gMC3. Our results also show that partially purified C3bINA is more effective in inactivating E1gMC3 if the exposure of E1gMC3 to this C3bINA occurs prior to monocyte-E1gMC3 association (Fig. 3). C3bINA in serum has a similar effect if its action on E1gMC3 is examined after a shorter E1gMC3 and C3bINA
interaction time (Table 1). These results suggest a subsequent monocyte-erythrocyte interaction that follows monocyte binding of ElgMC3.

Previous studies by Nussenzweig and co-workers suggested that activation of the alternative complement pathway can remove immune complexes bound to the surface of phagocytic cells. Our data with monocyte-bound erythrocytes clearly indicate that ElgMC3 displacement is independent of such a mechanism. The reaction proceeds in heat-inactivated serum, which destroys properdin factor B, and in the presence of partially purified C3bINA.

Thus these experiments in vitro implicate C3bINA in the displacement of ElgMC3 from the macrophage in IgM induced immune hemolysis. The reaction in vitro depends upon the concentration of C3 on the erythrocyte surface and on the level of C3bINA, suggesting that a similar relationship might exist in vivo.

REFERENCES


Effect of C3b inactivator on monocyte-bound C3-coated human erythrocytes

AD Schreiber and PB McDermott