Effect of Antigen Site and Complement Receptor Status on the Rate of Cleavage of C3c Antigen From Red Cell Bound C3b

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C3b was bound to human red cells when serum complement was activated by addition of antibodies directed against different red cell antigens, and the rate of cleavage to C3dg was determined by assay for loss of bound C3c antigens using radiolabeled monoclonal anti-C3c. When C3b was bound by antibodies to antigens on branched-chain glycoproteins, cleavage to C3dg occurred more rapidly than when C3b was bound by antibodies to antigens closer to the red cell lipid bilayer. The rate of cleavage to C3dg also correlated directly with the number of complement receptors (CR_1) per red cell, reflecting their role as cofactors in the cleavage of iC3b by factor I. Thus, the life span of C3b/iC3b on human red cells, which may be important for determining the rate and mechanism of clearance of C3-coated red cells, appears to depend on the CR_1 status of the red cells and the characteristics of the antigen sites around which complement is bound.

ANTIBODIES directed against human red cells vary considerably in their ability to shorten red cell survival. The ability of an antibody to activate complement and to fix C3b appears to be an important factor in determining its clinical effects, including intravascular hemolysis and increased binding to phagocytic cells.[1] On the other hand, C3dg-coated red cells may not demonstrate a shortened red cell survival.[2] Thus the degradation to C3dg is believed to play an important role in the regulation of red cell injury by complement.[1,2] Using antibodies to different red cell antigens to fix C3b, we determined that the rate of loss of the C3c moiety depends upon both the antigen site around which C3b is bound and the number of CR_1 per red cell.

METHODS

Blood from individuals with appropriate red cell antigenic phenotype was drawn into isotonic saline containing 1 mmol/L EDTA. The red cells were washed twice in Alsever's solution (ACD), once in veronal-buffered isotonic saline (VBS), once in VBS with .15 mmol/L Ca^{++} and .5 mmol/L Mg^{++} (VBS-M), and then resuspended in VBS-M. For comparison of two antigens the cells from a single individual with both antigens were used. Red cells from patients with hematologic disorders were obtained in accordance with policies of the Buffalo General Hospital and Durham Veterans Administration Medical Center Human Subjects Committees.

The antibodies used are listed in Table 1. A and I antigens are contained principally on long branched-chain glycoproteins of the red cell membrane.[3] IgG anti-A antibody was obtained by Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography of serum from a blood group O individual with IgG anti-A antibodies. The IgG-containing fractions were pooled and concentrated in a macrosolute concentrator (Amicon Corp, Danvers, MA). Anti-I was an IgM monoclonal antibody (MoAb) from a patient with chronic cold agglutinin disease (kindly supplied by Dr Wendell Rosse, Durham, NC). "Anti-Tj^a" (anti-PP_1P^k) was an IgM antibody from a patient with the p phenotype, which binds predominantly to the P antigen, a tetrasaccharide directly attached to membrane sphingolipid. Anti-D IgG alloantibody was obtained from individuals who had been immunized with D-positive red cells. The structure of the D antigen has not yet been determined, but membrane lipid appears to be important for its antigenicity. Because the D antigen is relatively sparse and complement activation by IgG antibody against this antigen generally does not occur, complement activation was induced in the region of the D antigen by adding affinity-purified rabbit antihuman IgG (Copper Bio Medical Inc, Malvern, PA) in an amplification step. Anti-Jk^a IgG alloantibody was obtained from serum of patients immunized by red cell transfusion (kindly provided by Dr Richard Plunkett, Buffalo, NY). The location and structure of the Jk^a antigen is unclear.

To induce complement activation by warm-reactive antibodies, human red cells in VBS-M were incubated at 37°C for ten minutes with dilutions of antibody and type-compatible fresh human serum as a source of complement and fluid-phase complement inhibitors. For the D antigen, cells were first incubated with primary IgG antibody in the absence of fresh serum, washed, incubated with affinity-purified rabbit antihuman IgG, washed again, and then incubated with fresh serum. For complement activation by the cold-reactive antibodies, anti-I and anti-Tj^a, red cells, antibody, and serum were mixed, and a biphasic incubation was used. The mixture was incubated at 4°C for five minutes, slowly warmed to 37°C over five minutes, and then incubated an additional five minutes at 37°C. Complement activation was terminated by the addition of 10 mmol/L EDTA.

To measure the amount of bound C3c antigen present over time, the cell suspension was incubated at 37°C, and aliquots were removed at timed intervals. Cells in the aliquots were immediately diluted 50-fold with VBS and washed three more times with VBS. The cell-bound C3c antigen that remained was quantitated by measuring the binding of mouse monoclonal antihuman C3c antibody (Bethesda Research Labs, Gaithersburg, MD) radiolabeled with ^{125}I using chloramine-T.[4] Fifty microliters of cell suspension was incubated at 37°C with 50 μL radiolabeled anti-C3c (3 ng/μL)

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Table 1. Characteristics of the Antibodies Used to Bind C3b to Red Cells

Antigens	Antibody Class	Thermal Amplitude	Antigen Localization
A	IgG	Warm	Long-chain glycoprotein or glycolipid
I	IgM	Cold	Long-chain glycoprotein or glycolipid
Jk^a	IgG	Warm	Structure unclear
Tj^a	IgM	Cold	Short-chain glycolipid
D	IgG	Warm	Short-chain lipoprotein

in triplicate over a 3:2 (vol/vol) mixture of n-butyl and bis (2-ethylhexyl) phthalate oils for 20 minutes. The cells were then centrifuged through the oil in a Beckman microfuge B, effecting separation of cell-bound from unbound anti-C3c. The cell-bound radioactivity in the tube tips was measured, and the amount of anti-C3c bound per cell was calculated. Anti-C3c binding was plotted against time to calculate the half-time ($t_{1/2}$) for the loss of C3c antigen from red cells. Since the anti-C3c bound to C3b and iC3b but not to C3dg or C3d, it was possible to determine the rate of degradation to C3dg. In experiments comparing the rates of degradation to C3dg at different antigenic sites, the final concentrations of red cells and fresh serum were held constant during the incubation following the addition of EDTA, usually at 2×10^8 cells/mL and 25% serum. At the concentrations used, the rates of degradation were maximal and relatively insensitive to small variations in the concentrations of red cells, serum, or EDTA.

Membrane CR_1 per red cell was measured using the binding of monoclonal E11 antibody to the human red cell complement receptor. The antibody was radiolabeled using chloramine-T. Red cells were adjusted to a concentration of approximately 2×10^8/mL in VBS, counted in duplicate using a model D-2N Coulter Counter, and 50-μL aliquots were incubated in triplicate with 50-μL aliquots of radiolabeled anti-CR_1 at 3 ng/μL for 30 minutes at 20°C. The cells were then centrifuged through the phthalate oil mixture and anti-CR_1 bound per cell calculated.

For the purpose of correlating mean CR_1 per red cell to factor I cofactor activity, C3b was bound to red cells using cold agglutinin antibody and degradation to C3dg allowed to occur, as described above. Control red cells were obtained weekly to standardize anti-CR_1 binding by comparison to cells stored for seven days at 4°C in ACD and to compare the $t_{1/2}$ of degradation in the cofactor activity assay.

RESULTS

Plots of anti-C3c bound on a logarithmic scale against time were linear, suggesting pseudo–first-order kinetics for a single rate-limiting interaction and allowing calculation of the $t_{1/2}$ for the loss of C3c antigen from the red cell surface. The results of one such experiment are shown in Fig 1. Although different amounts of C3b were initially bound to red cells by different concentrations of anti-I antibody, the $t_{1/2}$ for C3c loss was independent of the amount of initial C3b bound within the range shown and nearly identical for the different antibody concentrations used. Similar results were also obtained when different initial amounts of C3b were bound to red cells by different concentrations of anti-Tj^a. For initial binding of 3 and 8 fg/cell anti-C3c, incubation of appropriate cells with anti-Tj^a was associated with half-times of 38 and 42 minutes, respectively. With each antibody the rate of loss of C3c antigen was relatively constant and was not faster when more C3b was initially bound.

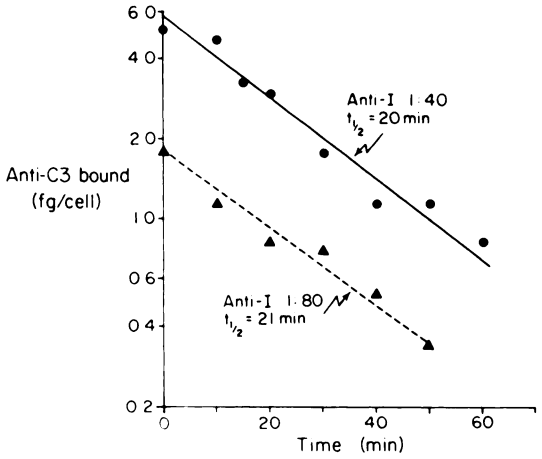

Fig 1. Cleavage of C3c antigen from C3b bound to red cells by anti-I antibody is shown. Different initial amounts of C3b were bound by varying the concentration of anti-I antibody used. The logarithm of anti-C3c binding to C3b/iC3b remaining is linear with respect to time, and the $t_{1/2}$ of degradation is independent of initial C3b bound.

Representative results of experiments in which C3c antigen was measured after complement was activated by antibody to A, D, I, and Jk^a antigens are shown in Fig 2. Rates of C3c antigen loss were similar after activation by anti-A and anti-I but were 2.5 and 3.0 times longer, respectively, when anti-D and anti-Jk^a were used. Therefore antibodies to A and I apparently bound C3b to red cells at sites that seemed to permit loss of C3c antigen to proceed at rates that were several times faster than rates seen when antibodies to D and Jk^a were used.

Whereas antibodies to A, D, and Jk^a were IgG antibodies, anti-I was a cold-reactive IgM antibody. To determine whether differences in the rates of loss of C3c antigen were properties related to the antibody used to activate comple-

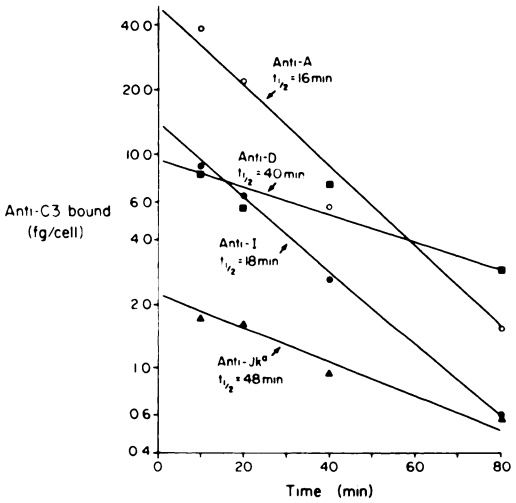

Fig 2. Cleavage of C3c antigen from C3b bound to red cells by different antibodies is shown. Degradation of C3b/iC3b deposited near A and I antigen sites is more rapid than that deposited near D or Jk^a.

ment, we compared them to the rates that were derived when complement was activated by anti-I and anti-Tj^a, both of which were cold-reactive IgM antibodies. There were marked differences in the rates of C3c antigen loss following C3b fixation by these two different IgM antibodies. In five separate experiments the $t_{1/2}$ for loss of C3c antigen from red cells treated with anti-Tj^a was nearly twice that seen when anti-I was used ($t_{1/2}$ of 35.3 ± .5 minutes when anti-Tj^a was used and 20.0 ± .2 minutes when anti-I was used). Thus it appeared that differences in the site of activation and deposition of C3b were affecting the subsequent rate of C3c antigen loss rather than differences in class or thermal amplitude of the antibody used to activate complement.

Decrease in anti-C3c binding over time could represent the decay or degradation of C3b/iC3b to C3dg or the release or loss of C3b/iC3b from the red cell surface. Release of covalently bound C3b has been demonstrated in fluid-phase systems.[5] To establish whether loss of C3c antigen following fixation of complement was derived from the degradation of C3b/iC3b or from shedding of the intact molecule, we measured C3dg that remained bound as C3c antigen was lost. Complement was activated by cold agglutinin antibody; the reaction was terminated by EDTA and followed for 90 minutes. Anti-I was chosen because complement activated by this cold agglutinin produced the shortest $t_{1/2}$ of loss of C3c antigen and therefore would be the one most affected by shedding. Bound C3c antigen was measured as described above, and bound C3dg was measured using radioiodinated monoclonal mouse anti-C3dg. The results are depicted in Fig 3. Over a period during which anti-C3c binding declined tenfold, anti-C3dg binding remained unchanged. Loss of C3c antigen did not reflect loss of C3dg antigen. Thus it appeared that there was no significant release of fixed C3b/iC3b during the period of observation in our assay system.

The red cell complement receptor CR_1 binds to C3b, iC3b, and C4b. It has been shown that CR_1 is the cofactor for the physiologic cleavage by factor I of bound iC3b to bound C3dg and fluid-phase C3c.[6-8] We examined the factor I cofactor activity (measured by the loss of C3c antigen) of red cells from 41 different individuals, including 30 patients with diseases associated with decreased red cell CR_1 number (connective tissue disorders and hematologic malignancies).[9,10] In such patients CR_1 molecules are lost from the red cell surface as a consequence of the disease process and are not usually blocked by autoantibodies or immune complexes. When complement was activated by cold agglutinin anti-I antibody, C3c was removed with a $t_{1/2}$ ranging from 200 to 20 minutes for cells that bound .05 to .30 fg/cell anti-CR_1 (approximately 100 to 1,000 molecules anti-CR_1/cell). Linear regression analysis of the log of the $t_{1/2}$ plotted as a function of anti-CR_1 binding per cell gave a line with a slope of −.6 and a correlation coefficient of .89 (Fig 4). Thus over a sixfold range of CR_1 number, the rate of degradation of C3b/iC3b to C3dg depended upon the number of CR_1 per red cell.

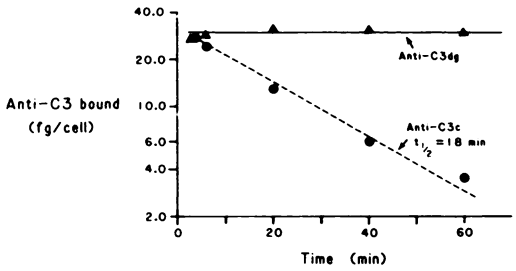

Fig 3. Persistence of C3dg antigen and cleavage of C3c antigen from C3b bound to red cells by anti-I antibodies is shown. Anti-C3dg binding remains unchanged as anti-C3c binding declines.

DISCUSSION

The location of membrane bound C3b is strongly influenced by the site of activation, and when complement is activated by antibody, this site is in the vicinity of the antigens to which the antibody is directed.[11] Native serum C3 undergoes an activating cleavage by the C3 convertase complexes of either the classic or alternative pathways of complement.[12] The cleavage results in liberation of a polypeptide fragment of C3, C3a, and produces activated or "nascent" C3b, which may bind covalently to nearby nucleophilic acceptor molecules with an ester or amide bond.[13] Nascent C3b has a short lifetime measured in microseconds and thus may diffuse on the order of 30 nm before becoming covalently bound.[14] If such a bond is not formed within that time, the reactive site in nascent C3b is hydrolyzed. We studied degradation of C3b bound by antibodies against antigens with varied membrane placement using radiolabeled monoclonal anti-C3c to detect bound C3c antigens. We determined that loss of C3c antigen represented cleavage rather than shedding of C3b/iC3b from red cells and required CR_1 cofactor activity.

The rates of loss of C3c depended upon the antigenic specificity of the antibodies used to activate complement. When complement was bound to red cells by antibodies to A and I, antigenic determinants contained principally on branched long-chain polysaccharides of red cell glycoproteins and glycolipids, C3b/iC3b appeared to be more readily degraded than when complement was bound as a result of antibody interacting with relatively membrane-confined

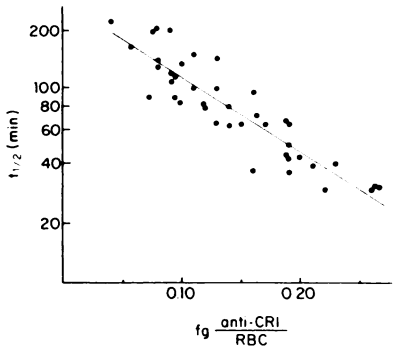

Fig 4. Relationship of the $t_{1/2}$ of degradation of bound C3b/iC3b to number of CR_1 per red cell is shown. There is an inverse linear correlation (r = 0.89) between the log of the $t_{1/2}$ of degradation and anti-CR_1 binding per cell.

antigens such as P and D. The P antigen is a tetrasaccharide attached directly to membrane sphingolipids, and the D antigen is thought to be a transmembrane protein requiring red cell lipid for antigenic integrity.[3] The C3b/iC3b bound to red cells by antibody to Jk^a was degraded at rates similar to that bound by antibody to P and D. Although the structure of the Jk^a antigen is not known, our results imply that its structure also renders C3b deposited in its vicinity less susceptible to degradation. When complement was activated by human anti-D IgG followed by rabbit antihuman IgG, it is possible that C3b was bound to antibody rather than to D antigen, thereby affecting the rate of degradation.

C3 is the central protein of the complement cascade and the formation, decay, and degradation of C3b or C3b-containing convertases is influenced and regulated by many factors, including decay accelerating factor,[15] factor H,[16] and factor I with CR_1 as cofactor. CR_1 has been shown to accelerate decay of classical pathway C3 convertases, to potentially serve as a cofactor for cleavage of C3b to iC3b by factor I, and to facilitate degradation of iC3b to C3dg.[17-20] In our experiments the concentration of factor I and factor H were held constant by using the same dilution of fresh serum as a source of complement regardless of the type or amount of antibody used to activate complement. In experiments with red cells having different amounts of CR_1 per cell, the concentration of factor I in serum was also maintained at a constant level. CR_1 cofactor activity therefore appeared to be rate limiting for the cleavage of C3b/iC3b to C3dg under the near-physiologic conditions employed. In experiments correlating the $t_{1/2}$ of C3c antigen loss and anti-CR_1 binding, we found degradation to C3dg depended upon CR_1. Thus the degradation of C3b/iC3b to C3dg occurred with pseudo–first-order kinetics, with a rate determined by the number of CR_1 per red cell and by the antigen site.

Since C3b fixation is restricted to the area of C3 convertase assembly, which is defined by the antigen site in classical pathway activation, the interpretation is that the ability of CR_1 to interact with C3b/iC3b varies with different antigen sites. There are several characteristics of C3b fixation around different antigen sites that may be important to variable interaction with CR_1 and especially the potential for multivalent interaction of multimeric CR_1 and C3b/iC3b on different cells. The first of these is clustering of C3b/iC3b. Clustered antigen sites might lead to deposition of C3b/iC3b clusters that were more readily available for multivalent CR_1 interaction. A related characteristic is the mobility in the membrane plane of the bound C3b/iC3b and its ability to interact with mobile CR_1 in the same or in adjacent cells. If the C3b/iC3b were bound to moieties fixed in the membrane plane, multivalent interaction would be more difficult, whereas if the moieties were flexible and/or mobile, multivalent interaction would be enhanced. A third characteristic might be steric accessibility to CR_1.[19] C3b/iC3b bound to exposed long-chain glycoproteins might be more accessible for interaction with CR_1 than C3b/iC3b bound to antigens nearer the lipid layer. Similar considerations might apply to accessibility of bound iC3b to factor I in the degradation of iC3b to C3dg.

Whatever the mechanisms affecting susceptibility of C3b/iC3b to cofactor activity of CR_1, our results suggest that the Jk^a and P antigen sites are physiologically different from the A or I antigen sites. These findings suggest that the Jk^a antigen itself or nearby available C3b binding sites are perhaps either less exposed, less mobile, or more sparse than those of the long-chain polysaccharide antigens. These differences in antigen characteristics may produce different effects in vivo. Because the subsequent cellular interactions (eg, with phagocytes or lymphocytes) might be different depending on the amount of C3b/iC3b as opposed to C3dg on a red cell, the antigen-dependent differences in C3b/iC3b cleavage may have important consequences for the clinical effects of anti-red cell antibodies.[1] The rate of degradation of C3b bound by antibodies to different antigens may be useful in defining antigen structure and localization. The availability of the C3b/iC3b binding sites for interaction with red cell CR_1 provides additional information about the complex and dynamic geography of membranes.

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