

COVALENT ASSOCIATION OF C3b WITH C4b WITHIN C5 CONVERTASE OF THE CLASSICAL COMPLEMENT PATHWAY

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The fifth component of complement (C5) is a source of two biologically important complement fragments, C5a and C5b. Both fragments have roles in elimination of targets of complement action. The smaller fragment, C5a, acts as a chemotactic factor in attracting polymorphonuclear and mononuclear phagocytes to the area, where complement activation is taking place (1). The bigger fragment, C5b, triggers the formation of the membrane-attack complex, C5b-9 (2, 3). The cleavage of C5 into these two fragments is mediated by a complex enzyme, C5 convertase. C5 convertase is assembled upon cleavage of C3 by C3 convertase.

In the classical pathway, C3 convertase is a noncovalently associated complex of C4b and C2a, C4b2a. Within C4b2a, C2a has a catalytic site and C4b anchors the complex to the target (4) and perhaps also acts as a binding site for the substrate, C3. The current concept about the assembly of the classical C5 convertase is as follows: C4b2a cleaves multiple molecules of C3 to C3a and nascent C3b molecules, and some of the latter become covalently bound to acceptors (hydroxyl and amino groups) on the surface of the target (5). Among the bound C3b, one that has been bound in the close proximity of C4b2a acts as a subunit of the C5 convertase by serving as a binding site for the substrate, C5 (6, 7). The convertase-forming C3b, however, is not distinguished from C3b molecules bound at a distance from C4b2a. In addition, it is unknown how a suitable trimolecular complex, which perhaps requires a somewhat defined quaternary structure, is formed accurately on a wide variety of targets on which the distribution of acceptors for nascent C3b may be random.

In the present study we examined the nature of the C5-binding site within the classical C5 convertase. In this report, we show that one of the nascent C3b molecules generated by the C3 convertase directly binds covalently to C4b and also present evidence that the C4b-C3b dimer thus formed serves as a high-affinity binding site for C5. In this way, C5 may bind selectively to the convertase in spite of surrounding monomeric C3b molecules.

This work was supported by grants from the Veritas Corporation, Tokyo; the Uehara Memorial Foundation; and by grant B-60480163 from the Ministry of Education, Science and Culture of Japan.

Materials and Methods

Buffers. The buffers used were isotonic veronal buffer, pH 7.4, containing 72.7 mM NaCl, 2.5% dextrose, 0.1% gelatin, 0.15 mM CaCl₂, and 1 mM MgCl₂ (DGVB);¹ 0.1% gelatin veronal-buffered saline, pH 7.4, containing 10 mM EDTA (EDTA-GVB); a sample buffer for SDS-PAGE composed of 80 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.005% bromphenol blue and 6 M urea (2-ME [5%] was added for reduction); and hypotonic lysing buffer made up of 5 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA.

Complement Components. Guinea pig C1 (8) and C2 (9), and human C4 (10), C3, and C5 (11) were purified as described. The protein concentrations of C3, C4, and C5 were determined by the method of Lowry et al. (12) with OVA (Seikagaku Kogyo Co., Tokyo, Japan) as a standard. The molecular weights used to calculate molar concentrations were: C3, 190,000; C3b, 180,000; C4, 210,000; C4b, 200,000; and C5, 200,000. C3, C4, and C5 were labeled with ¹²⁵I (Amersham Corp., Arlington Heights, IL) in Iodogen (Pierce Chemical Co., Rockford, IL)-coated test tubes (13). Free iodine was removed by dialysis. The preparations had specific activities of 1–5 × 10⁶ cpm/μg protein and were fully active after iodination.

Red Cell Hemolytic Intermediates. Antibody-sensitized sheep erythrocytes (EA) bearing C1 (EAC1) were prepared by incubation of EA cells (10⁸/ml) with guinea pig C1 (200 U/ml) for 15 min at 30°C. EAC14 cells were prepared by incubating EAC1 cells (10⁸/ml) with an equal volume of human C4 (either labeled with ¹²⁵I or unlabeled) at a concentration of 12–24 μg/ml for 30 min at 30°C. EAC142 cells were prepared by incubating EAC14 cells (10⁸/ml) with an equal volume of 400 U of guinea pig C2 for 10 min at 30°C. EAC1423 cells were prepared by incubating EAC142 cells (10⁸/ml) with an equal volume of human C3 (either labeled with ¹²⁵I or unlabeled) at a concentration between 1 and 50 μg/ml for 30 min at 30°C. The amounts of C4b and C3b deposited on EAC14 and EAC1423 cells were determined from the uptake of labeled C4 and C3. EAC43 cells were prepared by suspending EAC1423 cells in EDTA-GVB at 37°C for 2 h, and then washing them with DGVB. EAC4 cells were prepared by suspending EAC14 cells in EDTA-GVB at 37°C for 15 min, and then washing them with DGVB.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (14). Two-dimensional gel electrophoresis was carried out by the method of Law and Levine (15). The molecular weight marker proteins used were ferritin (220,000), phosphorylase b (94,000), BSA (67,000), and OVA (43,000) in reducing conditions; and mouse IgM (900,000), human C4-binding protein (560,000 and 500,000), and C4b (200,000) in nonreducing conditions.

Determinations of the Amounts of C4b-C3b Dimer, C4b Monomer, and C3b Monomer on EAC1423 Cells. EAC1423 cells bearing radiolabeled C4b or C3b were solubilized in SDS-PAGE nonreducing sample buffer and subjected to SDS-PAGE followed by autoradiography. The intensities of the bands of C4b-C3b dimer and the monomer on the autoradiogram were measured by densitometric analysis with a DU-8B Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The amounts of C4b-C3b dimer and monomer molecules were calculated from the ratio of these bands and the total amount of cell-bound C4b or C3b, which was calculated from the cell-bound radioactivity.

Assay of Binding of C5 to Sheep Cell Hemolytic Intermediates and Determination of the Number of C5-binding Sites. In the standard binding assay, samples of cell suspension (10⁸/ml) were incubated with equal volumes of increasing concentrations of radiolabeled C5 (12.5–2,000 ng/ml) in DGVB for 60 min at 30°C. Duplicate 40-μl samples from each reaction mixture were layered over 300 μl of a mixture of seven volumes of dibutylphthalate (Nakarai Chemicals, Kyoto, Japan) and three volumes of dinonylphthalate (Nakarai Chemicals) in 400 μl polypropylene microtest tubes (Sarstedt, Rommelsdorf, Federal Republic of Germany). After centrifugation for 3 min at 4°C in a Beckman B microcentrifuge (Beckman Instruments, Inc.) at 8,000 g, the tubes were cut just above the cell

¹ *Abbreviations used in this paper:* DGVB, half-isotonic gelatin veronal-buffered saline; EA, antibody-sensitized sheep erythrocytes; EAC1, EA bearing C1; EDTA-GVB, gelatin veronal-buffered saline containing EDTA.

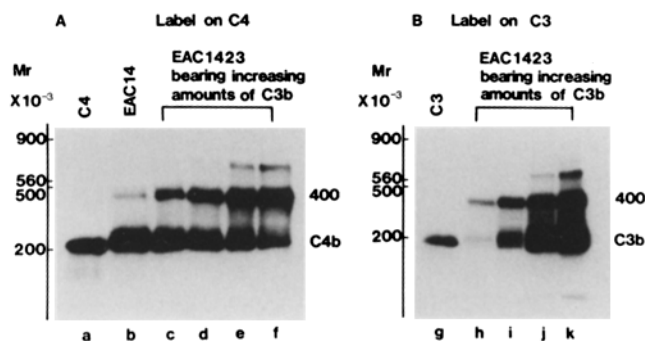


FIGURE 1. SDS-PAGE analysis under nonreducing conditions of EAC1423 cells bearing radiolabeled C4b or radiolabeled C3b. EAC1423 cells bearing a constant amount of C4b and increasing amounts of C3b were solubilized in SDS-PAGE nonreducing sample buffer and the samples were analyzed by SDS-PAGE on 4.5% gels followed by autoradiography. (A) Radiolabeled C4 and unlabeled C3 were used; (B) unlabeled C4

and radiolabeled C3 were used; (a) intact free C4; (b) EAC14 bearing 25,000 C4b/cell; (c and h) EAC1423 bearing 12,000 C3b per cell; (d and i) EAC1423 bearing 33,000 C3b per cell; (e and j) EAC1423 bearing 88,000 C3b/cell; (f and k) EAC1423 bearing 220,000 C3b per cell; (g) intact free C3. The band at the 400,000- M_r position increased as a result of binding of C3b.

pellets and the radioactivity of bound C5 in the sections containing the pellets was measured directly. The amount of free C5 was calculated by subtracting the radioactivity in the pellets from the total radioactivity. Data obtained by the binding assay were analyzed by a Scatchard-plot (16) to determine the number of C5-binding sites and the association constant.

Determination of the Half-Life of the Bond between C4b and C3b within the C4b-C3b Dimer. The amount of cell-bound C4b-C3b dimer decreases due to cleavage of the bond between either C4b and C3b or C4b and a cell-surface acceptor. Therefore, the half-life of cell-bound C4b-C3b dimer is given by: (a) $T = 1n [2/(K_1 + K_2)]$ where T is the half-life of the dimer and K_1 and K_2 are the rate constants of cleavages of the bonds between C4b and cell-surface acceptors and between C4b and C3b, respectively. K_1 and K_2 are given by: (b) $K_1 = 1n [2/T_1]$, (c) $K_2 = 1n [2/T_2]$ where T_1 and T_2 are the half-lives of the bonds between C4b and cell-surface acceptors and between C4b and C3b, respectively. From equations (a), (b), and (c) we arrive at the equation: (d) $T_2 = TT_1/(T_1 - T)$. Because T and T_1 can be determined experimentally, T_2 , the half-life of the bond between C4b and C3b, can be calculated from equation d.

Results

Formation of C4b-C3b Dimer on EAC1423. When EAC14 cells bearing radiolabeled C4b were analyzed by SDS-PAGE under nonreducing conditions, a major band with approximately the same mobility as free C4 and a faint band at the 400,000- M_r position were seen (Fig. 1b). The pattern of the bands was consistent with that for the C4B isotype described by Isenman and Young (17), i.e., most of the C4b was associated with membrane constituents with low molecular weight and a small amount of C4b was associated with 200,000- M_r acceptor molecules. The EAC14 cells were converted to EAC142 cells and then incubated with increasing amounts of unlabeled C3. The resulting set of EAC1423 cells bearing a constant amount of C4b and increasing amounts of C3b was analyzed by SDS-PAGE. As shown in lanes c-f, a band at the 400,000- M_r position appeared and increased in intensity with increase of C3b. Concomitantly, the intensity of the main band of C4b decreased. Because the total amount of C4b was constant, the results indicated a shift of C4b from the 200,000- to the 400,000- M_r position with increase in the amount of C3b binding. We interpreted this result as indicating that C4b served as an acceptor molecule for nascent C3b forming

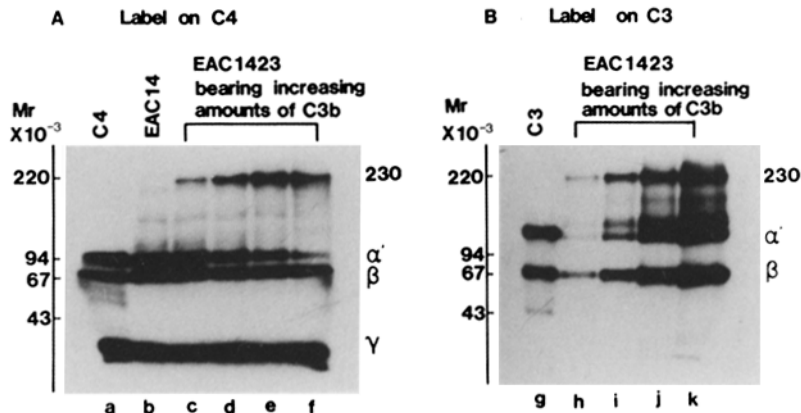


FIGURE 2. SDS-PAGE analysis under reducing conditions of EAC1423 cells. EAC1423 cells were prepared as described in the legend of Fig. 1. The samples were analyzed by SDS-PAGE on 7.5% gels under reducing conditions and autoradiographed. (A) Radiolabeled C4 and unlabeled C3 were used; (B) unlabeled C4 and radiolabeled C3 were used; (a) intact free C4; (b) EAC14 bearing 25,000 C4b per cell; (c and h) EAC1423 bearing 12,000 C3b per cell; (d and i) EAC1423 bearing 33,000 C3b per cell; (e and j) EAC1423 bearing 88,000 C3b per cell; (f and k) EAC1423 bearing 220,000 C3b per cell; (g) intact free C3. The band at the 230,000- M_r position appeared and increased as a result of binding of C3b.

covalently bound C4b-C3b dimer. For confirmation of this point, a similar experiment was performed with radiolabeled C3 and unlabeled C4. As shown in lanes h-k, the band at the 400,000- M_r position was also demonstrated with C3 radiolabel, supporting the idea that this band contains C4b and C3b. Considering the size of the band, one molecule of C3b probably binds to one molecule of C4b. A faint band at the ~600,000- M_r position was also seen when a large amount of C3b was bound (lanes e, f, j, and k). This band may represent a trimer consisting of one molecule of C4b and two molecules of C3b.

Fig. 2 shows the results of analysis of the same sets of EAC1423 cells under reducing conditions. As shown in lanes b-f, increasing amounts of C4b- α' chain band shifted from the 94,000- M_r to the 230,000- M_r position with increase in the binding of C3b. The band at the 230,000- M_r position was also demonstrated with C3 radiolabel (lanes h-k). These results suggest that the C3b- α' chain (110,000 M_r), which had contained the metastable binding site to an acceptor, became associated with the C4b- α' chain (85,000 M_r), which had been bound to a low-molecular-weight acceptor molecule.

It should be pointed out that the formation of the C4b-C3b dimer is a quite efficient reaction. For example, densitometric analysis of the autoradiogram shown in Fig. 1B showed that 54% of the bound C3b was associated with C4b in lane h, in which the ratio of C3b to C4b bound per cell was ~1:2. The proportion of C4b-associated C3b decreased with increase in the amount of bound C3b, as seen in Fig. 1, lanes i-k.

We used immunoprecipitation with anti-C4 antibodies to demonstrate the association of C3b with C4b by a different method. EAC1423 cells (60,000 molecules of C4b and 115,000 molecules of C3b per cell) prepared with radiolabeled C3b were lysed hypotonically and the ghosts were extracted with 1% NP-40. Anti-C4 IgG plus Sepharose-protein A precipitated 55% of C3 radiolabel

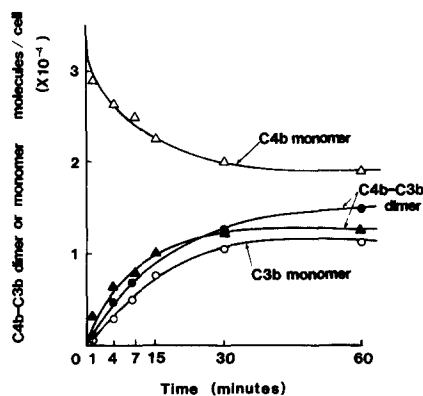


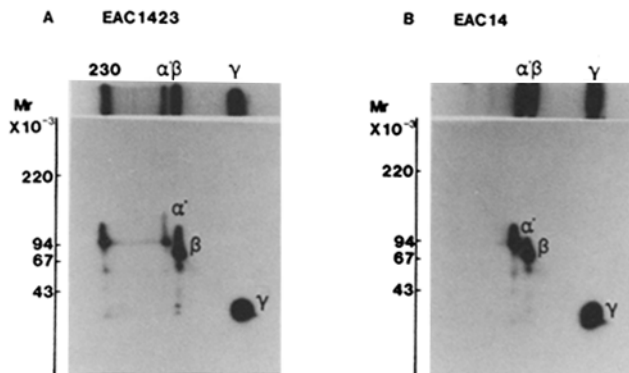
FIGURE 3. Time course of formation of C4b-C3b dimer on EAC1423 cells. EAC142 cells (4×10^7) bearing 33,000 C4b per cell were incubated with $2 \mu\text{g}$ of C3 in $800 \mu\text{l}$ of DGVB at 30°C . Radiolabeled C4 and unlabeled C3 were used in one group (Δ , \blacktriangle), and unlabeled C4 and radiolabeled C3 in the other (\circ , \bullet). At the indicated times, aliquots of cells were taken from each mixture. The cells were promptly washed, solubilized in SDS-PAGE nonreducing sample buffer, and subjected to SDS-PAGE on 4.5% gel followed by autoradiography. The amount of C4b-C3b dimer at each time (\blacktriangle , \bullet) was determined by densitometric measurement of the intensity of the band at the $400,000\text{-}M_r$ position. The amounts of monomer C4b (Δ) and C3b (\circ) were assessed in a similar way by measuring the intensities of the bands at the $200,000\text{-}M_r$ position on the respective autoradiograms. C4b-C3b dimer was formed quite efficiently.

from the extract, whereas normal IgG precipitated 0.3% of the radioactivity. When the precipitate was dissolved in SDS-PAGE sample buffer and analyzed by SDS-PAGE, only the band at $400,000\text{-}M_r$ position was shown under nonreducing conditions (data not shown). These results further support the notion that a significant part of C3b binds directly to C4b to form a C4b-C3b dimer.

Next we studied the time course of the formation of the C4b-C3b dimer (Fig. 3). When EAC142 cells bearing radiolabeled C4b were incubated with unlabeled C3, the amount of C4b-C3b dimer (*filled triangles*) increased with concomitant decrease of C4b monomer (*open triangles*). The reaction reached a plateau level after 30 min incubation, probably because of decay dissociation of C2a. Next, unlabeled C4 and radiolabeled C3 were used, and a rate of formation of C4b-C3b dimer (*filled circles*) was compared with that of formation of cell-bound C3b monomer (*open circles*). As seen in the figure, dimer formation proceeded at a rate similar to or even faster than C3b monomer formation. Therefore, the formation of C4b-C3b dimer is an efficient process, and accordingly, C4b is a quite reactive acceptor molecule for nascent C3b.

Nature of the Covalent Linkage between C4b and C3b. The experiment shown in Fig. 2 suggested the presence of a binding site(s) for C3b on the α' chain of C4b. A preliminary experiment showed that the bond between C4b and C3b is hydrolyzed by hydroxylamine. Therefore, two-dimensional SDS-PAGE was used to demonstrate directly the ester-mediated binding of C3b to the α' chain of C4b (Fig. 4). For this, EAC1423 cells bearing radiolabeled C4b and unlabeled C3b were subjected to first-dimension electrophoresis under reducing conditions to separate the dimeric band of $230,000 M_r$ from the monomeric α' chain, and then after hydroxylamine treatment, electrophoresis in the second dimension was carried out. As shown in Figure 4A, the dimeric band at the $230,000\text{-}M_r$ position was completely dissociated by hydroxylamine treatment and the α' chain of C4b appeared at an off-diagonal position, indicating that C3b had been bound to the α' chain of C4b by an ester linkage.

Next we studied the stability of the ester bond between C4b and C3b. EAC1423 cells were incubated at pH 7.4 and 37°C , and the amounts of cell-bound total C4b and C4b-C3b dimer were determined at timed intervals for 8



sample buffer, and then placed on top of a 7.5% gel for electrophoresis in the second dimension. As a control, EAC14 cells were treated similarly. The autoradiograms are shown. (A) EAC1423; (B) EAC14. Autoradiograms of identical but untreated strips from the first dimension are shown horizontally on the top of each panel for comparison. The band at the 230,000 M_r position seen after electrophoresis in the first dimension was completely dissociated by treatment with hydroxylamine with appearance of the α' chain of C4b.

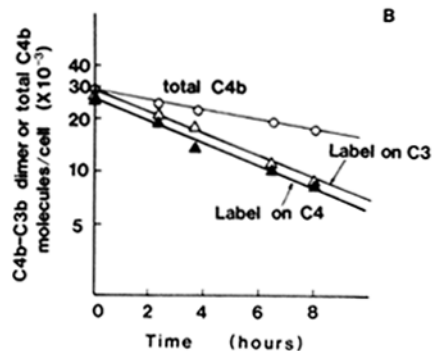
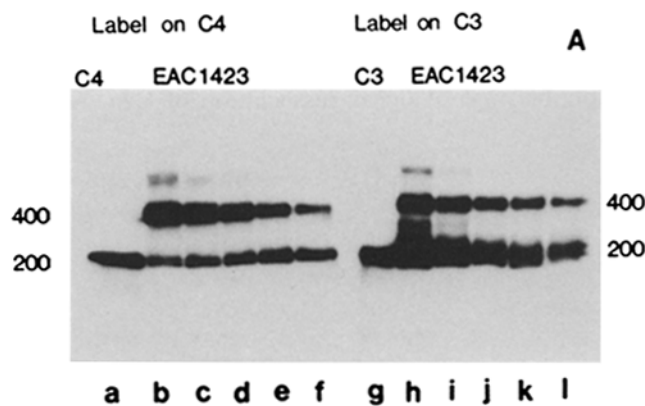


FIGURE 4. Analysis of C4b-C3b dimer by two dimensional SDS-PAGE. EAC1423 cells bearing 25,000 radiolabeled C4b and 220,000 unlabeled C3b per cell were solubilized in SDS-PAGE sample buffer and separated in the first dimension by SDS-PAGE on 7.5% gel under reducing conditions. The gel strip was incubated for 60 min at 37°C in 1 M hydroxylamine in 0.1 M NaHCO₃ at pH 9.5 to cleave ester bonds, reequilibrated with SDS-PAGE reducing

FIGURE 5. Stability of C4b-C3b dimer. EAC1423 cells (4×10^7) bearing either radiolabeled C4b (29,000 C4b per cell) or radiolabeled C3b (106,000 C3b per cell) were incubated at 37°C in 800 μ l of EDTA-GVB. At the indicated times, 100- μ l aliquots were taken, and the cells were washed with EDTA-GVB, solubilized in SDS-PAGE nonreducing sample buffer, and analyzed by SDS-PAGE. (A) Autoradiogram of EAC1423 prepared with radiolabeled C4 (left) or radiolabeled C3 (right). (b and h) Before incubation at 37°C; (c and i) after 37°C incubation for 2.3 h, (d and j) 3.7 h, (e and k) 6.5, and (f and l) 8 h. (a) Intact C4; (g) intact C3. $M_r \times 10^{-3}$ are shown. (B) Amounts of C4b-C3b dimer determined by densitometric measurement of the autoradiogram plotted as a function of time. (\blacktriangle) radiolabel on C4, (\triangle) radiolabel on C3. The total amount of C4b determined from cell-bound total radioactivity is also plotted (O). The decrease of C4b-C3b dimer and total C4b both showed first-order kinetics.

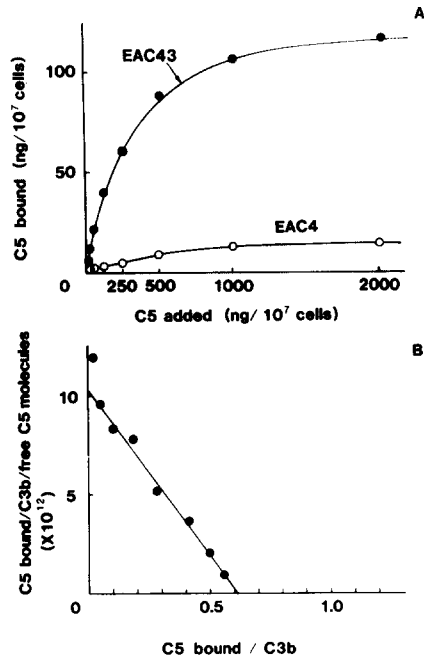


FIGURE 6. Quantitative analysis of binding of C5 to EAC43 cells. Increasing amounts of radiolabeled C5 (16–2,000 ng) were incubated for 60 min at 30°C with 10^7 EAC43 (63,000 C4b and 75,000 C3b per cell), EAC4 (63,000 C4b), or EA cells in 200 μ l of DGVB. After incubation, the cells were separated from unbound C5 by centrifugation through oil and the radioactivities of the pellets were determined. Specific binding of C5 to EAC43 or EAC4 cells was calculated by subtracting the radioactivity bound to EA cells from the radioactivity bound to these cells. (A) The amounts of radiolabeled C5 bound to EAC43 (●) or EAC4 (○); (B) a Scatchard plot of binding of C5 to EAC43. Results showed the presence of a high-affinity binding site for C5 with an association constant of 2.1×10^8 L/M. 1.6 molecules of C3b were required for one binding site.

h (Fig. 5). As shown by the radioautogram in Fig. 5A, the intensity of the band of C4b-C3b dimer decreased with time. In B, the amounts of cell-bound C4b-C3b dimer and total C4b are plotted as functions of time. As can be seen, the degradations of both C4b-C3b dimer and total C4b followed first-order kinetics, the half-lives being 4.7 and 11.7 h, respectively. Since cell-bound C4b-C3b dimer could have been degraded by cleavage of the bond between either C4b and C3b or C4b and a cell-surface acceptor, the half-life of the ester bond between C4b and C3b was calculated to be 7.9 h from the equation (d) described in Materials and Methods. This result shows that the bond between C4b and the cell-surface acceptor is more stable than that between C4b and C3b. Therefore, a certain amount of cell-bound monomeric C4b was probably formed as a result of the degradation of the C4b-C3b dimer. Indeed, the intensity of the band of monomeric C4b gradually increased with time (Fig. 5A, left half). In contrast, the intensity of the bands of monomeric C3b decreased with time (Fig. 5A, right half).

Studies on the C5-binding Site within C5 Convertase of the Classical Pathway. The above results indicate that there are two kinds of C3b on EAC1423 cells, one covalently bound to C4b and the other bound to cell-surface constituents. To determine whether only the former C3b served as a binding site for C5, we first studied the binding of C5 to EAC43 cells. EAC43 cells were used to avoid any possible effect of cleavage of C5 by C2a. In Fig. 6A, the amounts of C5 bound specifically to EAC43 and to EAC4 are plotted as functions of added C5. Saturable binding curves were obtained with both types of cells. The binding curve with EAC43 cells was analyzed by Scatchard-plot, and as shown in Fig. 6B, the results indicated a homogeneous high-affinity binding site with an association constant of 2.1×10^8 L/M. The number of the high-affinity binding sites per

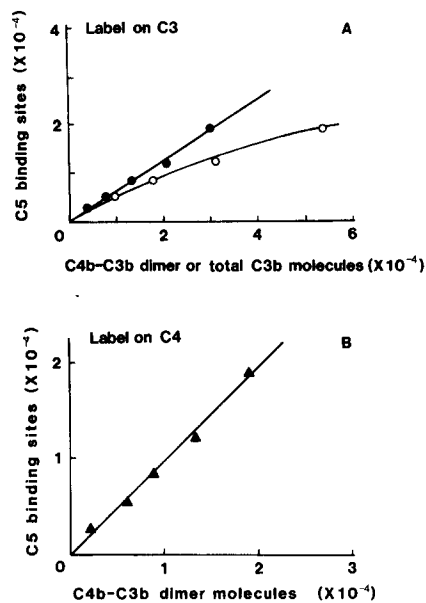


FIGURE 7. Relationship between C5-binding sites and the C4b-C3b dimer. Three sets of EAC43 cells bearing 30,000 C4b per cell and increasing amounts of C3b (4,000; 9,700; 18,000; 31,000; and 53,000 C3b per cell) were prepared. One set of cells was prepared with unlabeled C4 and C3, and used to measure the number of C5-binding sites. The number of C5-binding sites was determined by Scatchard-plot analysis as described in Materials and Methods. Specific binding of C5 to EAC43 cells was calculated by subtracting the number of C5 bound to EAC4 cells bearing 30,000 C4b per cell. The other two sets of cells, prepared with radiolabeled C4 and radiolabeled C3, respectively, were used to measure the number of C4b-C3b dimer molecules. The number of C4b-C3b dimer molecules was determined by SDS-PAGE and densitometric analysis, as described in Materials and Methods. (A) the number of C5-binding sites is plotted as a function of the total number of C3b molecules (○) or C4b-C3b dimer molecules (●), determined with radiolabeled C3; (B) the number of C5-binding sites is plotted as a function of the number of C4b-C3b dimer molecules, determined with radiolabeled C4. The amount of dimer probably decreased several percent during the incubation with C5 because of instability of the dimer (Fig. 5), but values are not corrected. The number of C5-binding sites was in good agreement with the number of dimer molecules.

molecule of C3b was determined to be 0.61, i.e., one C5-binding site per 1.6 molecules of C3b. Because this result indicates that only part of the bound C3b acts as a high-affinity binding site for C5, we next tested whether the number of C4b-C3b dimers coincides with the number of C5-binding sites on EAC43 cells. To obtain the results in Fig. 7, EAC43 cells bearing various amounts of C4b-C3b dimer were prepared and the number of C5-binding sites and the number of C4b-C3b dimers on these cells were determined. As shown in panel A, a plot of the number of the binding sites as a function of the number of dimers, which was determined using radiolabeled C3, showed a linear relationship (*filled circles*). The ratio of the number of the sites to the number of dimers was 0.65. In contrast, a linear relationship was not seen between the number of sites and the total number of cell-bound C3b molecules (*open circles*). For Fig. 7B, the number of dimers was determined with C4 radiolabel. Again, a linear relation was observed between the number of sites and the number of dimers. In this case, the ratio of sites to dimers was $\sim 1:1$. These results strongly suggest that C3b that binds directly to C4b makes a high-affinity binding site for C5.

As another method to examine the relationship between the number of binding sites and the number of dimers, a set of EAC43 cells bearing various amounts of C4b-C3b dimer was prepared by incubating EAC1423 cells at 37°C for various times of 0–8 h to allow degradation of the dimer. As shown in Fig. 8A, the amount of C5 bound to these cells decreased with increase in the incubation time. In B, the number of C5-binding sites (*open triangles*) and the number of C4b-C3b dimer molecules (*filled triangles*) are plotted as functions of the time of incubation. The two straight lines obtained by these plots coincide almost completely, again showing close coincidence of these two numbers.

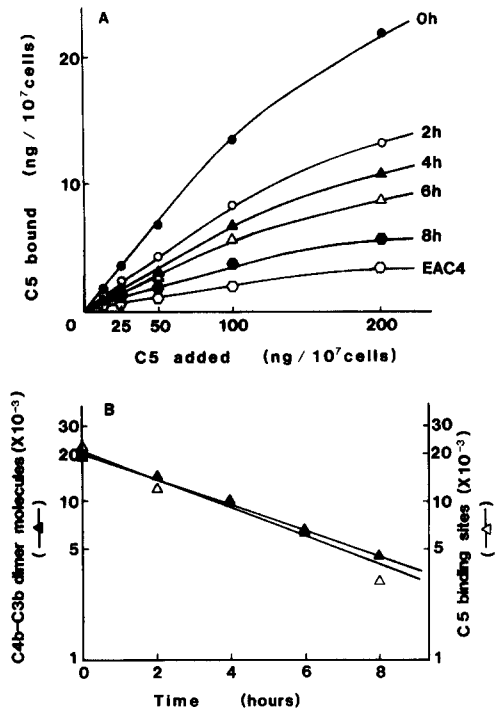


FIGURE 8. Stability of C5-binding sites on EAC1423. Two sets of EAC1423 cells (4×10^7) bearing 40,000 C4b per cell and 56,000 C3b per cell were incubated at 37°C in 800 μ l of EDTA-GVB for times shown on figure. One set of cells, which was prepared with unlabeled C4 and C3, was incubated with increasing amounts of radiolabeled C5 to measure the number of C5-binding sites. The other set of cells, bearing radiolabeled C4b, was used to measure the number of C4b-C3b dimer molecules. (A) The amounts of C5 binding to preincubated EAC1423 and EAC4 cells. The numbers of C5-binding sites per EAC43 were determined by Scatchard-plot analysis as described in Materials and Methods. The amount of C5 binding decreased with the time of preincubation of EAC1423 cells. The amounts of C4b-C3b dimer molecules on preincubated EAC1423 cells were determined by SDS-PAGE and densitometric analysis as described in Materials and Methods. (B) The number of C5-binding sites (Δ) and the number of C4b-C3b dimer molecules (\blacktriangle) on the preincubated EAC1423 are plotted as functions of time of incubation. The decreases of the two were similar, indicating a close coincidence of the two numbers.

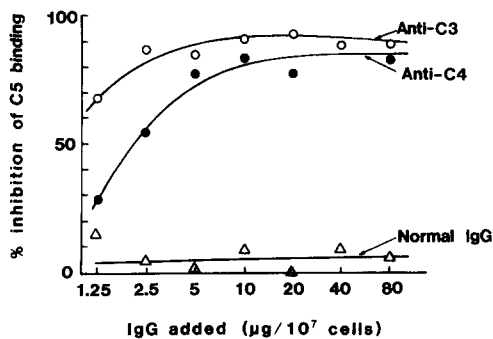


FIGURE 9. Inhibition of the binding of C5 to EAC43 cells by anti-C4 antibodies. EAC43 cells (10^7) bearing 23,000 C4b and 37,000 C3b per cell were incubated with increasing amounts of anti-C4, anti-C3, or normal IgG (0–80 μ g) in 200 μ l of DGVB for 10 min at 37°C. Then the cells were washed and incubated with 100 ng of radiolabeled C5 for 60 min at 30°C. A set of EA cells was similarly treated as controls. Specific binding of C5 to EAC43 was determined as described in the legend of Fig. 6, and the percentage inhibition by antibodies of the binding of C5 was calculated as follows: Percent inhibition = $100 \times [1 - (\text{specific binding to IgG treated EAC43}) / (\text{specific binding to untreated EAC43})]$.

Next we tested whether anti-C4 antibodies inhibited the binding of C5 to EAC43 cells, because if C5 binds only to C3b that is bound directly to C4b, antibodies to C4 should inhibit the binding of C5. As shown in Fig. 9, anti-C4 IgG caused 80% inhibition of the binding of C5.

Studies on the C5-binding Sites on EAC14 Cells. Since the experiment shown in Fig. 6 demonstrated binding of C5 to EAC4 cells, we studied the nature of the C5-binding sites on cells not bearing C3b. Fig. 10A shows the binding curve of C5 with EAC14 cells. Scatchard-plot analysis of the data (B) indicated a high-affinity binding site with an association constant of 8.1×10^7 L/M. The number of binding sites per molecule of C4b was 0.01, i.e., 1 binding site per 100 molecules of C4b.

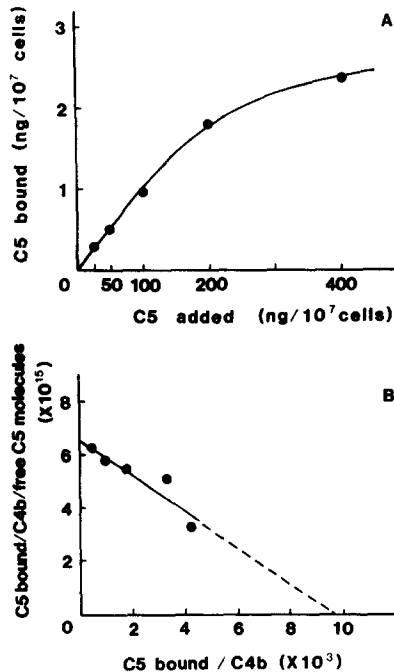


FIGURE 10. Quantitative analysis of binding of C5 to EAC14 cells. Increasing amounts of radiolabeled C5 (25–400 ng) were incubated for 60 min at 30°C with 10⁷ EAC14 cells bearing 180,000 C4b per cell or EAC1 cells as a control in a total volume of 200 μ l. After incubation, 40- μ l aliquots were overlaid on 300 μ l of oil and centrifuged, and the radioactivity in the pellets was determined. Specific binding of C5 to EAC14 cells was calculated by subtracting the binding to EAC1 cells. (A) A plot of the amount of specific C5 binding as a function of C5 input; (B) a Scatchard plot of the same data. The results indicated a high-affinity binding site with an association constant of 8.1×10^7 L/M. ~100 molecules of C4b were required to make 1 binding site.

Anti-C4 but not anti-C3 antibodies were found to inhibit the binding of C5 to EAC14 cells (data not shown). This result excludes the possibility of contamination of the cells with C3 and suggests that C5 indeed binds to C4b.

Because 100 molecules of C4b were necessary for 1 binding site, it seems likely that C4b, which binds directly to cell-bound C4b, serves as the binding site for C5. When EAC14 cells prepared with radiolabeled C4 were analyzed by SDS-PAGE, faint bands were seen at the 400,000- M_r position under nonreducing conditions (Fig. 1, lane *b*) and at the 200,000- M_r position under reducing conditions (Fig. 2, lane *b*). From their size and intensities, the bands seem to represent those of the C4b-C4b dimer and C4b α' -C4b α' dimer, respectively.

Discussion

One of the main findings in this study was that among the multiple molecules of nascent C3b, which are generated by the classical pathway C3 convertase, one becomes covalently bound to C4b with formation of a C4b-C3b dimer. Analysis of EAC1423 cells by SDS-PAGE demonstrated the presence of the covalently associated dimer of C4b and C3b (Figs. 1 and 2). An immunoprecipitation experiment also demonstrated the presence of C4b-associated C3b; i.e., anti-C4 antibodies precipitated a large amount of C3b from a detergent extract of EAC1423 cells. In SDS-PAGE of the precipitate, the band of C4b-C3b dimer was seen as a major population. Formation of the dimer was a quite efficient reaction; e.g., 54% of the cell-bound C3b was associated with C4b when the ratio of C3b to C4b bound per cell was small (~1:2). Kinetic analysis also indicated efficient formation of the C4b-C3b dimer; the rate of dimer formation was similar to or even faster than that of formation of cell-bound monomeric C3b

molecules (Fig. 3). These results indicate that C4b is a highly reactive acceptor molecule for nascent C3b and suggest the presence of some affinity between the two proteins that facilitates formation of the covalent linkage.

We found by SDS-PAGE analysis that C3b binds to the α' chain of C4b within the C4b-C3b dimer. Since the dimer was dissociated completely by treatment with hydroxylamine, the bond between C4b and C3b was mostly of the ester type (Fig. 4). C3b probably binds to a specific hydroxyl group on the α' chain of C4b, rather than binding in a random fashion, because C5 convertase may have a defined quaternary structure and because the affinity between C4b and nascent C3b may fix nascent C3b to a specific site on C4b. Consistent with this idea that C3b binds to a specific hydroxyl group on the α' chain of C4b, the ester bonds between the protomers were found to be homogeneous with respect to their half-life. As shown in Fig. 5, although the bond between C3b and C4b was covalent, it was rather unstable, with a half-life of 7.9 h at pH 7.4 and 37°C. This half-life was slightly shorter than that of the bond between C4b and the cell-surface acceptor (11.7 h), and was one-third of that reported for the complex of C3b and glycerol (~24 h, reference 18).

Another major finding in this study was that EAC43 cells have high-affinity C5-binding sites with an association constant of 2.1×10^8 L/M (Fig. 6). The results in Figs. 7-9 provided evidence that only C4b-associated C3b serves to make a high-affinity binding site for C5; namely, they showed that the number of high-affinity C5-binding sites coincides with the number of C4b-C3b dimer molecules and that anti-C4 antibodies effectively inhibit the binding of C5 to EAC43 cells. Monomeric C3b does not seem to serve as a high-affinity binding site. The association constant for binding between monomeric C3b and C5 is probably much lower and may be similar to those of 5×10^6 L/M and 2×10^6 L/M reported for cell-bound C3b (19) and fluid-phase C3b (20), respectively. The simplest explanation for the big difference between the affinities of monomeric C3b and C4b-associated C3b may be that C5 associates with the latter at two sites, one on C3b and the other on C4b. The site on C3b would be the same as the site on monomeric C3b that has low affinity. The putative site on C4b may also have low affinity, but higher avidity should be obtained by two-site binding.

Our results indicated that C5 binds effectively to C4b-associated C3b. Probably, among the bound C3b molecules the C4b-associated C3b molecule is the nearest to the catalytic site on C2a. Most of the other surrounding monomeric C3b molecules may not be suitable as a subunit of the C5 convertase because they are either too far from C2a or are in a wrong direction, or both. The high-affinity of the C4b-C3b dimer for C5 allows selective binding of C5 to the C3b nearest to the catalytic site. Therefore, the classical pathway C5 convertase could be described as a trimolecular complex in which C2a noncovalently associates with the C4b portion of the covalently linked C4b-C3b dimer.

The surfaces of various targets of the complement are heterogeneous with respect to the density and distribution of hydroxyl and amino groups, and fluidity. Nevertheless, C5 convertase can be formed reliably on any type of targets, once C4b has bound, because C3b binds directly to C4b. Since, as discussed above, C3b probably binds to a specific site on C4b, the quaternary

structure of the C4b-C3b dimer may be defined, and this in turn may allow defined presentation of the substrate, C5, to the catalytic site of C2a.

A small amount of high-affinity C5 binding sites was demonstrated on EAC14 cells. Probably, a small fraction of nascent C4b (~1 in 100 molecules) becomes associated with bound-C4b to form the C4b-C4b dimer. Since C4b has a great deal of homology to C3b (21), it is not surprising that C5 associates with the C4b-C4b dimer with high affinity. There is a report that EAC14^{oxy2} cells are lysed in C5-C9 without participation of C3 (22), suggesting that C4bC2aC4b might act as a C5 convertase. Interestingly, sera from patients with C3 deficiency have some hemolytic activity and this is C3-independent (23). Taken together, these findings suggest that C4b can replace C3b as a subunit of the classical C5 convertase.

The classical and alternative pathways are similar in many respects (24–26). Therefore, it seems reasonable to suppose that C3b associates directly with Bb-bearing C3b within the alternative pathway C5 convertase. Consistent with this idea, we recently demonstrated the presence of a covalently associated dimer of C3b on rabbit erythrocytes after their incubation with human serum, and the presence of high-affinity C5-binding sites on sheep erythrocytes bearing alternative pathway C5 convertases (our unpublished observations). Further characterization of the alternative C5 convertase is in progress.

Summary

The C5 convertase of the classical complement pathway is a complex enzyme consisting of three complement fragments, C4b, C2a, and C3b. Previous studies have elucidated functional roles of each subunit (4, 6, 7), but little is known about how the subunits associate with each other. In this investigation, we studied the nature of the classical C5 convertase that was assembled on sheep erythrocytes. We found that one of the nascent C3b molecules that had been generated by the C3 convertase directly bound covalently to C4b. C3b bound to the α' chain of C4b through an ester bond, which could be cleaved by treatment with hydroxylamine. The ester bond was rather unstable, with a half-life of 7.9 h at pH 7.4 and 37°C. Formation of the C4b-C3b dimer is quite efficient; e.g., 54% of the cell-bound C3b was associated with C4b when 25,000 molecules of C4b and 12,000 molecules of C3b were present per cell. Kinetic analysis also showed the efficient formation of the C4b-C3b dimer; the rate of dimer formation was similar to or even faster than that of cell-bound monomeric C3b molecules. These results indicate that C4b is a highly reactive acceptor molecule for nascent C3b.

High-affinity C5-binding sites with an association constant of 2.1×10^8 L/M were demonstrated on C4b-C3b dimer-bearing sheep erythrocytes, EAC43 cells. The number of high-affinity C5-binding sites coincided with the number of C4b-C3b dimers, but not with the total number of cell-bound C3b molecules. Anti-C4 antibodies caused 80% inhibition of the binding of C5 to EAC43 cells. These results suggest that only C4b-associated C3b serves as a high-affinity C5 binding site. EAC14 cells had a small amount of high-affinity C5 binding sites with an association constant of 8.1×10^7 L/M, 100 molecules of bound C4b being necessary for 1 binding site. In accordance with the hypothesis that C4b-

associated C4b might also serve as a high-affinity C5-binding site, a small amount of C4b-C4b dimer was detected on EAC14 cells by SDS-PAGE analysis. Taken together, these observations indicate that the high-affinity binding of C5 is probably divalent, in that C5 recognizes both protomers in the dimers. The high-affinity binding may allow selective binding of C5 to the convertase in spite of surrounding monomeric C3b molecules.

We thank Dr. Takachika Azuma for discussions and Keiko Kinoshita for technical assistance.

Received for publication 9 February 1987.

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