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Formation of High-Affinity C5 Convertases of the Alternative Pathway of Complement[1]

Nenoo Rawal[2] and Michael K. Pangburn

Cleavage of C5 by C5 convertase is the last enzymatic step in the complement activation cascade leading to the formation of the cytolytic proteolytically activated form of C5 (C5b)-9 complex. In the present study, we examined the effect of the density of C3b (the proteolytically activated form of C3) on the function of the noncatalytic subunit of natural surface-bound forms of the enzyme. A comparison of the kinetic parameters of C5 convertases assembled on three surfaces (zymosan, rabbit erythrocytes, and sheep erythrocytes) were similar and revealed that the average K_m decreased ~28-fold (5.2–0.18 μM) when the density of C3b was increased from ~18,000 to 400,000 C3b/cell. Very-high-affinity C5 convertases were generated when preformed C3 convertases were allowed to self amplify by giving them excess C3. These convertases exhibited K_m from 0.016 to 0.074 μM, well below the normal plasma concentration of C5 in blood (0.37 μM). The results suggest that in serum convertases formed with monomeric C3b will be relatively inefficient in capturing C5 but will continue to cleave C3 opsonizing the cell surface for phagocytosis, whereas convertases formed with C3b-C3b complexes in areas of high C3b density will primarily cleave C5. The catalytic rate of these convertases approaches maximum velocity, thereby switching the enzyme from cleavage of C3 to cleavage of C5, and production of the cytolytic C5b-9 complex. ***The Journal of Immunology,*** **2001, 166: 2635–2642.**

Activation of complement via the innate and the adaptive immune systems is vital to the body's defenses in fighting infections. Complement activation triggers a series of reactions that involves >30 different proteins (1–3). These proteins are activated in a sequential manner resulting in the generation of products that have important biological activity. Cleavage of C5, the fifth component of complement, by the serine protease C5 convertase is an important enzymatic reaction that occurs during the activation cascade. C5 convertase cleaves C5 into two products, C5a and C5b (1). Both fragments play a vital role in killing microorganisms and in eliminating targets of complement action. C5b, the larger fragment, initiates the assembly of the cytolytic (C5b-9) complex, which results in the lysis of bacteria and other microorganisms. C5a, the smaller fragment, is a potent chemotactic and spasmogenic anaphylatoxin that mediates inflammatory responses by stimulating platelets, endothelial cells, eosinophils, neutrophils, and phagocytes to the site of complement activation (4, 5).

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[3] Abbreviations used in this paper: C3b, proteolytically activated form of C3; E_C, chicken erythrocytes; E_R, rabbit erythrocytes; E_S, sheep erythrocytes; VBS, Veronal-buffered saline; GVB, gelatin VBS; GVBE, GVB containing 10 mM EDTA; NHS, normal human serum; ZymC3b,Bb, surface-bound C5 convertase assembled on zymosan particles; E_RC3b,Bb, surface-bound C5 convertase assembled on E_R; E_SC3b,Bb, surface-bound C5 convertase assembled on E_S; C3b,Bb, monomeric C3/C5 convertase assembled with monomeric C3b; V_{max}, maximum velocity; k_{cat}, catalytic rate constant.

C5 convertases of the alternative and classical pathways of complement are complex serine proteases that are made up of two or more subunits (1, 6–11). The study of C5 convertases has been difficult because the enzyme complex that cleaves C5 is the same serine protease (C3 convertase) that cleaves C3 (1). Cleavage of C3 produces the proteolytically activated form of C3 (C3b),[3] which forms the nonenzymatic subunit of the enzyme complex responsible for C3 and C5 cleavage (1, 3). C3 convertases of the alternative pathway (monomeric C3/C5 convertase assembled with monomeric C3b (C3b,Bb)) and the classical pathway (C4b,C2a) are bimolecular complexes (1, 3). The alternative pathway C3 convertase (C3b,Bb) has C3b as the noncatalytic subunit attached in a Mg^{2+}-dependent fashion to the catalytic subunit (Bb) of the enzyme complex. In the classical pathway C3 convertase C4b,C2a has a structure similar to the alternative pathway C3 convertase in having C2a as the catalytic subunit noncovalently bound to a C4b molecule. The soluble serine protease C3 convertase (C3b,Bb) assembled with monomeric C3b has been shown to be the simplest form of C5 convertase (12). The natural surface-bound C5 convertases are the more complex forms of the enzyme and are made up of a C3 convertase and an additional C3b molecule (6–11, 13, 14). Attachment of the additional C3b molecules near the C3 convertase was originally thought to be required for generating C5 convertase activity (6, 8, 15). Vogt et al. (13) revealed that the role of the additional C3b was to bind the substrate, C5. Later a more specific structure was described. High C5 convertase activity was associated with the formation of C3b-C3b or C3b-C4b dimers in which the additional C3b molecule was shown to be covalently attached to a specific site on the first C3b or C4b molecule (10, 16). The dimers were suggested to form complexes C3b-C4b,C2a and $C3b_2$,Bb, which are currently considered to be functional C5 convertases of the classical and the alternative pathways, respectively (9, 10, 17, 18).

To enhance our understanding of the structure/function of natural surface-bound C5 convertases we examined the effect of C3b density on the functional role of the noncatalytic subunit of surface-bound C5 convertases. In this study we show that, whereas the density of C3b/cell influences the K_m and not the catalytic rate

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constant (k_{cat}) of C5 convertases, the type of surfaces do not influence either the K_m or the k_{cat} of the enzyme. The data suggest that the initial monomeric C3/C5 convertases formed on the surface because of their weak affinity for C5 will function as C3 convertases amplifying C3b around them. As C3b deposition occurs, C3b-C3b complexes are formed. These complexes form convertases that have higher affinities for C5 and will primarily cleave C5 leading to a higher production of the cytolytic C5b-9 complex. Based on the present kinetic analysis of the natural surface-bound C5 convertases together with results published by other groups on the structure of C5 convertase, we propose a model for the formation of high-affinity C5 convertases during complement activation.

Materials and Methods

Reagents

Chicken erythrocytes (E_C) were isolated from chicken blood purchased from Colorado Serum (Denver, CO). Nonidet P-40, a nonionic detergent, and EDTA were purchased from Sigma (St. Louis, MO). Veronal-buffered saline (VBS) contained 5 mM barbital and 145 mM NaCl (pH 7.4). Gelatin VBS (GVB) was VBS containing 0.1% gelatin, while GVB containing 10 mM EDTA (GVBE) was also used.

Purified proteins

Complement proteins, C3 (19, 20), factor B (21), factor D (22), and C6 (23) were all purified from normal human plasma as described in the references cited. C5 was also isolated from normal human plasma as described (24) except that ceramic hydroxylapatite (Bio-Rad, Richmond, CA) was used instead of hydroxylapatite. C3b was prepared from C3 by cleavage with factors B and D in the presence of Ni^{2+} at 37°C as described previously (12). Purified C5b,6 was obtained from Advanced Research Technologies (San Diego, CA). All proteins were homogenous by polyacrylamide gel electrophoresis. Protein concentrations of C3b, C5, C6, factor B, factor D, and C5b,6 were determined spectrophotometrically using 11.0, 11.0, 10.8, 12.7, 11.0, and 10.3, as the values for $E^{1\%}_{280\ nm}$, respectively. All purified proteins were stored at −76°C. M_r values employed in the calculations were 176,000 for C3b, 190,000 for C5, 179,000 for C5b, 120,000 for C6, 299,000 for C5b,6, 93,000 for factor B, 63,000 for Bb, and 24,000 for factor D.

Preparation of ZymC3b, E_RC3b, and E_SC3b

ZymC3b was prepared as described previously (12). C3b was deposited on zymosan by resuspending 1×10^{10} zymosan particles in 0.2 ml of 10 mg/ml C3 and adding 5 μg of trypsin, followed by a 10-min incubation at 22°C. The deposition of C3b by trypsin was repeated and the cells were washed six times with 5 ml GVB. The zymosan particles were resuspended in 100 μl GVB and mixed with 50 μl GVB containing factors B (35 μg) and D (0.5 μg) and 50 μl of 10 mM $NiCl_2$. After 5 min of incubation at 22°C, 5 μl of 0.2 M EDTA was added. The bound C3b was amplified by adding 50 μl C3 (500 μg) and incubating the cells for 30 min at 22°C. The zymosan particles bearing C3b were washed and the amplification procedure was repeated until the desired numbers of C3b/zymosan were obtained (25). The total number of C3b molecules bound to zymosan was measured by two independent methods using ^{125}I-labeled C3 (26) or radiolabeled factor B (12) as described previously. The number of C3b deposited on the zymosan particles was adjusted so as to obtain different densities of C3b/cell ranging from 18,000 to 405,000 C3b/cell. E_SC3b and E_RC3b bearing different amounts of C3b were prepared similarly.

Formation and assay of surface-bound C5 convertases

Because formation of C5 convertase took less than 1 min, enzyme was formed in the same reaction mixture in which the assays were performed. Enzyme velocities were determined under saturating concentrations of factors B and D and C6 in 0.5 ml siliconized microfuge tubes as described previously (12). Assay mixtures contained varying concentrations of C5 (preincubated for 20 min at 37°C to eliminate freeze/thaw-generated background C5b,6-like activity), factor B (1.2 μg, 516 nM), factor D (0.1 μg, 167 nM), C6 (2.5 μg, 833 nM), and 0.5 mM $NiCl_2$. The reaction was started by the addition of ZymC3b, E_SC3b, or E_RC3b. Depending on the density of C3b per cell, the concentration of cells was adjusted so as to have ~9–35 ng of bound C3b in a final volume of 25 μl GVB resulting in 2–8 nM enzyme concentration. After 15 min of incubation at 37°C, further cleavage of C5 was prevented by transferring the assay tubes to an ice bath and adding ice-cold GVBE. Appropriately diluted assay mixtures were immediately titrated for C5b,6 formation by hemolytic assays using E_C. C5b,6 was quantitated using standard curves generated with purified C5b,6. Controls established that the cold temperature and the dilution were sufficient to reduce the cleavage of C5 during subsequent steps to undetectable levels. Lysis of rabbit erythrocytes (E_R) or sheep erythrocytes (E_S) was shown to contribute <2% to C5b,6 titers using lysis of E_C as the endpoint.

Quantitation of reaction products

C5b,6 was measured hemolytically using the sensitivity of E_C to hemolytic lysis by human C5b-9 as described (12). To an aliquot (25 μl) of the diluted sample from C5 convertase assays was added a mixture of 1.2×10^7 E_C and 5 μl of pooled normal human serum (NHS) as a source of complement proteins C7-C9 in a final volume of 225 μl GVBE. The reaction mixtures were incubated for 10 min at 37°C after which the unlysed cells were removed by centrifugation for 1 min at 10,000 × *g*. The amount of hemoglobin released was quantitated spectrophotometrically at 414 nm. One-hundred percent lysis was measured as E_C lysed in 2% Nonidet P-40. Controls containing C5 and C6 but no C5 convertase, were subtracted as the background. Controls containing C5 convertase but no purified C5 or C6 demonstrated that no significant amount of C5b,6 was formed from NHS used as a source of C7–9 during the lysis of E_C.

Data analysis

The reaction velocity data was analyzed according to the Michaelis-Menten equation: $v = (V_{max})\,[S]/(K_m + [S])$. The results were fit to this equation using nonlinear regression analysis and the kinetic parameters, K_m, V_{max}, and k_{cat} were determined using Grafit version 4.12 software (Erithacus Software, London, U.K.).

Preparation of ^{125}I-labeled factor B and C3

Factor B (100 μg) was radiolabeled with ^{125}I for 30 min at 0°C in a glass tube coated with Iodogen (Pierce, Rockford, IL). Free ^{125}I was removed by centrifugal desalting (27). Specific activities of radiolabeled factor B ranged from 0.12 to 0.95 μCi/μg. C3 was labeled as described for factor B. The radiolabeled C3 was diluted with cold C3 to give a specific activity of 0.08 μCi/μg.

Determination of the number of C3b molecules bound per cell

Cells were amplified with C3b using radiolabeled C3 at 9.8 mg/ml (specific activity = 0.08 μCi/μg) instead of unlabeled C3 as described above. The amplification procedure was repeated to obtain cells bearing different densities of radiolabeled C3b ranging from ~18,000 to 700,000 C3b/cell. E_R bearing radiolabeled C3b were incubated for 5 min at 37°C in 25 μl GVB after which the reaction mixture was diluted with 75 μl GVB containing 0.5 mM $NiCl_2$. Bound and free radiolabel was separated by layering 75 μl of the mixture on 250 μl of 20% sucrose in GVB containing 0.5 mM $NiCl_2$ followed by centrifugation for 1 min at 10,000 × *g* at 22°C. The amount of radiolabeled C3b bound to cells was determined by cutting the tube and counting the amount of radioactivity in the pellet (26). The number of C3b bound to E_R calculated from the radioactivity (cpm) bound to E_R, is reported after subtracting nonspecific binding to control E_R.

Determination of the number of Bb binding sites (C5 convertase sites)

The number of C5 convertase sites formed on ZymC3b, E_RC3b, or E_SC3b was determined by measuring only those C3b molecules that were capable of forming an enzyme with radiolabel factor B under saturating assay conditions (12). ZymC3b, E_RC3b, or E_SC3b employed in the binding assays were washed three times with GVB to remove any free C3b. Various amounts of ^{125}I-labeled factor B were added to assay mixtures containing factor D (0.1 μg, 167 nM), C5 (5 μg, 1053 nM), C6 (2.5 μg, 833 nM), and 0.5 mM $NiCl_2$. Formation of enzyme was started with the addition of ZymC3b, E_RC3b, or E_SC3b to give a final volume of 25 μl GVB. After 5 min of incubation at 37°C, assays were diluted with 75 μl GVB containing 0.5 mM $NiCl_2$. Bound and free radiolabel were separated by layering 75 μl of the mixture on 250 μl of 20% sucrose in GVB containing 0.5 mM $NiCl_2$ followed by centrifugation for 1 min at 10,000 × *g* at 22°C. The amount of factor B bound to ZymC3b as Bb was determined by cutting the tube and counting the amount of radioactivity in the pellet (26).

Results

Determination of the number of cell-bound C3b and the number of Bb binding sites

To count the number of C3b/cell, E_R cells bearing different amounts of C3b ranging from ~18,000 to 700,000 C3b/cell were prepared using radiolabeled C3. The number of C5 convertase sites that could be generated on these E_R cells bearing different densities of radiolabeled C3b was determined as the number of Bb binding sites. Only those C3b molecules capable of binding ^{125}I-labeled factor B as Bb under saturating concentrations of factor D, Ni^{2+}, and radiolabeled factor B were quantitated. The specific activity of ^{125}I-labeled C3 (0.079 μCi/μg) was adjusted such that it was ~9-fold less than that of ^{125}I-labeled factor B (0.70 μCi/μg). This was necessary so as to have a 3-fold increase in the radioactivity over the background from C3b if all the bound C3b molecules could be saturated with Bb (12). The assays employed Ni^{2+} to minimize the decay of the Bb subunit of the enzyme during the 1-min spin through cold 20% sucrose. Quantitation of the number of C3b/cell and the number of Bb binding sites was done at the same time to minimize the possible effect of C3b decay from the cell surface. As seen in Fig. 1, the ratio of the number of Bb binding sites to the number of C3b/cell did not vary significantly with a 35-fold variation in density of C3b. The average ratio of Bb per C3b was determined to be 0.83 ± 0.13, (i.e., one Bb per 1.2 molecules of C3b) suggesting that most of the C3b can bind Bb even when the C3b density is greater than 400,000 C3b/cell. Given the errors associated with determining radioactive and specific activities of the two proteins, these results suggest that equivalent numbers of C3b and Bb bind at all levels of C3b/cell. Therefore, throughout this study we have determined the number of C5 convertase sites formed by measuring Bb binding under saturating levels of factor B and used it also as a measure of the number of C3b bound per cell.

Measurement of kinetic parameters of C5 convertases formed on activators of the alternative pathway of complement

Zymosan particles bearing C3b ranging from 18,600 to 405,000 C3b/Zym were employed for assembling C5 convertase. Enzyme activity was measured as the amount of C5 cleaved by quantitating the amount of C5b,6 formed during a 15-min assay under saturating concentrations of factors B and D and C6. Initial velocities were determined at various concentrations of C5. Data for ZymC3b,Bb made with 31,000 and 300,000 C3b/Zym are shown in Fig. 2, whereas data obtained with ZymC3b,Bb formed with other densities of C3b/cell are summarized in Fig. 3*A* and in Table I. The velocity data obtained were found to fit well to the theoretical curve based on the Michaelis-Menten equation: $v = (V_{max})[S]/(K_m + [S])$ (Fig. 2*A*). The data were also observed to fit well to the linearized form of the Michaelis-Menten equation, shown as the Eadie-Hofstee plot (Fig. 2*B*). C5 convertase formed with 300,000 C3b/cell had a 10-fold lower K_m (0.3 μM) than the enzyme formed with 31,000 C3b/cell (2.8 μM). These results suggest that the density of C3b/cell influences the K_m of the enzyme. The effect of the density of C3b/cell on the K_m of C5 convertase is apparent in the results shown in Fig. 3*A*, which illustrates a spectrum of different K_m values depending on the number of C3b/cell employed to assemble the enzyme. The average K_m of the enzyme decreased from 5.15 μM at 18,600 C3b/cell to 0.177 μM at

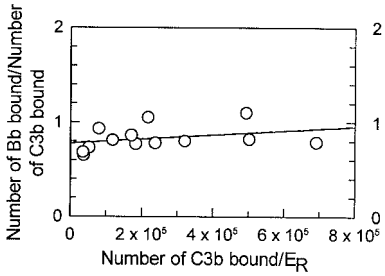

FIGURE 1. Comparison of the number of cell-bound C3b vs the number of Bb binding sites (C5 convertase sites). The number of C3b bound to the cell surface was determined by quantitating the amount of radiolabeled ^{125}I-labeled C3b bound to E_R cells. ^{125}I-labeled C3b was first deposited on E_R by trypsinization of radiolabeled C3 (0.8×10^5 cpm/μg) and then amplified with radiolabeled C3 as described in *Materials and Methods*. The number of cell-bound C3b was determined by incubating E_R ^{125}I-labeled C3b for 5 min at 37°C followed by centrifugation through 20% (w/v) sucrose. The amount of cell-bound ^{125}I-labeled C3b capable of forming a convertase was next determined by quantitating the amount of radiolabeled ^{125}I-labeled Bb bound to E_R bearing ^{125}I-labeled C3b. The binding assay was performed by incubating excess factors D and radiolabeled B (7.0×10^5 cpm/μg) and 0.5 mM $NiCl_2$ for 5 min at 37°C. The amount of ^{125}I-labeled Bb bound to E_R was calculated from the radioactivity in the pellet after spinning the E_RC3b, Bb cells through 20% sucrose and subtracting the contribution from ^{125}I-labeled C3b.

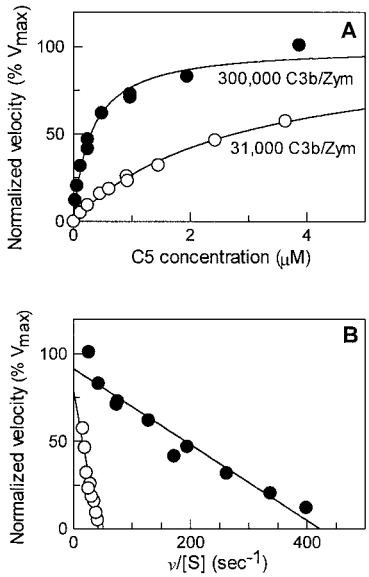

FIGURE 2. Kinetic analysis of C5 convertases formed on activators (zymosan particles) bearing 31,000 and 300,000 C3b/cell. *A*, Initial rates of C5 cleavage by C5 convertase, ZymC3b,Bb, were determined by measuring the amount of C5b,6 formed in 15 min at 37°C. Reactions contained saturating levels of factor B (0.6 μg, 258 nM), factor D (0.1 μg, 167 nM), C6 (2.5 μg, 833 nM), 0.5 mM $NiCl_2$, and the indicated concentrations of C5 in a final volume of 25 μl GVB. Reactions were initiated by addition of ZymC3b. For C5 convertases assembled with ZymC3b bearing 31,000 C3b/cell, 38.6 ng of C3b were present in the assay, which resulted in 8.8 nM of enzyme, whereas for those formed with 300,000 C3b/cell ZymC3b, 18.7 ng of C3b were present in the assay resulting in 4.25 nM enzyme. After 15 min at 37°C, the reactions were diluted with cold GVBE and analyzed for C5b,6 formation. An aliquot of 25 μl was quantitated for C5b,6 formation by adding 225 μl GVBE containing 1.2×10^7 E_C and 5 μl of pooled NHS as a source of C7-C9 to the reaction mixture. The assay mixture was incubated for 10 min at 37°C and the extent of hemolysis determined from the release of hemoglobin at 414 nm. The data were fitted by nonlinear regression according to the Michaelis-Menten equation to determine the K_m and V_{max} values using Grafit version 4.12 Erithacus software. The *y* axis represents the velocities that have been normalized by dividing by V_{max} and multiplying by 100. *B*, Analysis of the initial velocity data of the C5 convertases using the Eadie-Hofstee function.

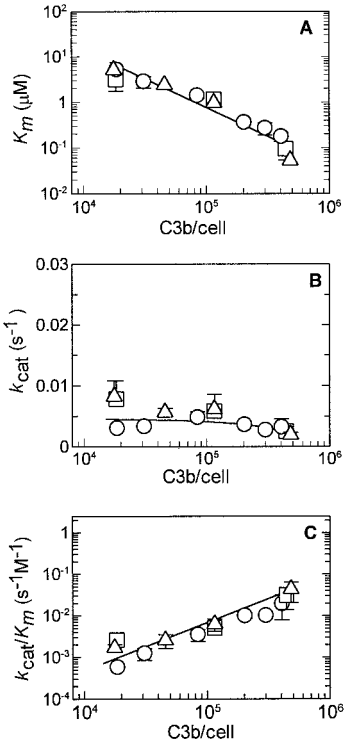

FIGURE 3. Comparison of the kinetic properties of C5 convertases assembled on three different surfaces (zymosan, E_R, and E_S) bearing different amounts of C3b. *A*, Comparison of the K_m of C5 convertases ZymC3b,Bb (○), E_RC3b,Bb (□), and E_SC3b,Bb (△) formed with cells bearing varying amounts of C3b/cell as indicated. The number of C3b/cell was measured as the number of Bb binding sites as described in Fig. 1. Initial velocities of C5 cleavage by C5 convertases assembled with varying C3b/cell were measured as the amount of C5b,6 formed at 37°C as described in Fig. 2. The K_m of each C5 convertase was determined from individual velocity vs substrate concentration plots. *B*, Comparison of the k_{cat} of C5 convertases ZymC3b,Bb (○), E_RC3b,Bb (□) and E_SC3b,Bb (△) with the number of C3b/cell as indicated. The k_{cat} for each C5 convertase was calculated from the V_{max} obtained from individual velocity vs substrate concentration plots and the enzyme concentration determined from the number of ^{125}I-labeled Bb bound/cell under saturating concentrations of factor B. The data obtained with all the three surfaces at the various indicated densities of C3b per cell were considered as one set of data points and were fitted by linear regression using Grafit version 4.12 Erithacus software. *C*, Comparison of k_{cat}/K_m ratio of C5 convertases ZymC3b,Bb (○) for E_RC3b,Bb (□) and for E_SC3b,Bb (△) with the number of C3b per cell as indicated. The k_{cat}/K_m ratio was calculated from the data in Table I.

405,000 C3b/cell (Table I). These results suggest that stronger binding interactions between the enzyme complex and the substrate C5 occurred with increasing C3b density. In contrast to the K_m, which varied widely, the catalytic rate (k_{cat}) of C5 convertases varied comparitively little at different densities of C3b/cell (Fig. 3*B* and Table I).

Determination of the kinetic constants of C5 convertases assembled on another activator, E_R bearing various amounts of C3b/cell indicated properties similar to that observed with the enzyme formed on zymosan particles. As seen in Fig. 3*A* and Table I, the K_m of E_RC3b,Bb decreased 33-fold from 3.14 μM at 18,600 C3b/cell to 0.095 μM at 434,000 C3b/cell while the catalytic rate of the enzyme decreased slightly by ~3-fold. These results suggest that the C3b density influences the K_m of C5 convertase but the type of surface employed for assembling the enzyme does not influence the K_m or the k_{cat} of C5 convertase.

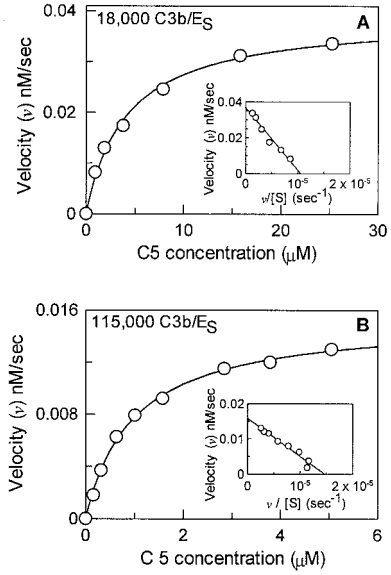

FIGURE 4. Kinetic analysis of C5 convertases formed on nonactivators (E_S) bearing 18,000 and 115,000 C3b/cell. *A*, Initial rates of C5 cleavage by E_SC3b,Bb assembled with 18,000 C3b per cell were determined as described in Fig. 2 except that E_SC3b bearing 46.7 ng C3b were added to the assay resulting in 10.6 nM of enzyme under saturating assay conditions. *Inset*, Analysis of the initial velocity of C5 convertase using the Eadie-Hofstee function. *B*, Velocity vs substrate concentration plot for E_SC3b,Bb assembled with 115,000 C3b/cell is shown. Assay conditions were as described in Fig. 2 except that E_SC3b bearing 14.5 ng C3b were added to the assay resulting in 3.3 nM of enzyme. *Inset*, Analysis of the initial velocity of C5 convertase using the Eadie-Hofstee function.

Measurement of kinetic parameters of C5 convertases formed on E_S, a nonactivator of the alternative pathway of complement

Although the data obtained with zymosan and E_R, both activators of the alternative pathway of complement, indicated that the surface does not influence the K_m of the enzyme we thought it important to examine the properties of C5 convertase formed on a nonactivating surface (E_S). The enzyme assembled on E_S exhibited kinetic properties similar to those obtained on the two activators of the alternative pathway of complement (Fig. 3 and Table I). The velocity vs substrate plots for E_SC3b,Bb assembled with E_S bearing 18,000 and 115,000 C3b/cell (Fig. 4, *A* and *B*, respectively) showed an excellent fit to the theoretical curve based on the Michaelis-Menten equation as well as to the linearized form of the equation (Eadie-Hofstee plot) shown as insets in Fig. 4. Thus, while the K_m of E_SC3b,Bb varied significantly (100-fold) depending on the density of C3b/cell, the k_{cat} of the enzyme varied slightly, decreasing by ~4-fold when the C3b density was increased from 18,000 to 479,000 C3b/E_S (Table. I).

Measurement of the kinetic parameters of self-amplified C5 convertases

The conversion of low affinity to high-affinity C5 convertases was studied by examining the enzymatic properties of self-amplified C5 convertases. These were the highest affinity C5 convertases that were generated in this study. They were prepared by allowing preformed C3 convertases to amplify C3b around themselves in the presence of excess C3. After amplification, the cells were washed and the convertases that had not yet decayed were assayed for C5 cleavage. The velocity data obtained were found to fit well to the theoretical curve based on the Michaelis-Menten equation (Fig. 5). The data were also observed to fit well to the linearized form of the

Table I. *Kinetic parameters of C5 convertases*[a]

Enzyme	C3b/cell[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Turnover Number (min^{-1})
Surface-bound C5 convertases					
ZymC3b,Bb	18,600*	5.2	0.003	0.6×10^3	0.18
	31,000	2.8 ± 0.8	0.0033 ± 0.0001	$1.2 \pm 0.4 \times 10^3$	0.12 ± 0.01
	84,000	1.4 ± 0.2	0.0048 ± 0.0009	$3.6 \pm 0.1 \times 10^3$	0.29 ± 0.06
	202,000	0.36 ± 0.01	0.0036 ± 0.0005	$10 \pm 1.0 \times 10^3$	0.22 ± 0.03
	300,000	0.27 ± 0.08	0.0027 ± 0.0006	$10 \pm 1.0 \times 10^3$	0.16 ± 0.04
	405,000	0.18 ± 0.04	0.0032 ± 0.0013	$20 \pm 12 \times 10^3$	0.19 ± 0.08
E_RC3b, Bb	18,000	3.1 ± 1.4	0.0078 ± 0.0030	$2.6 \pm 0.6 \times 10^3$	0.47 ± 0.18
	115,000*	1.2	0.0057	5.0×10^3	0.34
	434,030	0.095 ± 0.041	0.0025 ± 0.0004	$31 \pm 17 \times 10^3$	0.15 ± 0.02
E_SC3b,Bb	18,000	5.1 ± 2.2	0.0082 ± 0.0026	$1.7 \pm 0.3 \times 10^3$	0.49 ± 0.16
	45,000	2.4 ± 0.7	0.0056 ± 0.0007	$2.6 \pm 0.9 \times 10^3$	0.33 ± 0.04
	115,000	0.96 ± 0.22	0.0061 ± 0.0025	$6.2 \pm 2.1 \times 10^3$	0.36 ± 0.15
	479,000	0.053 ± 0.027	0.0019 ± 0.0004	$43 \pm 22 \times 10^3$	0.11 ± 0.01
Self-amplified C5 convertases					
ZymC3b,Bb		0.048 ± 0.006	ND		
E_RC3b,Bb		0.074 ± 0.028	ND		
E_SC3b,Bb		0.016 ± 0.007	ND		
Soluble monomeric C5 convertases					
C3b,Bb	1	24 ± 6.6	0.0110 ± 0.0038	$0.5 \pm 0.2 \times 10^3$	0.68 ± 0.22
CVF^h,Bb	None	14 ± 6.6	0.0121 ± 0.0061	$0.6 \pm 0.4 \times 10^3$	0.73 ± 0.37
CVF^n,Bb	None	0.036 ± 0.013	0.0071 ± 0.0026	$197 \pm 67 \times 10^3$	0.43 ± 0.16

[a] Kinetic parameters of surface-bound C5 convertases were determined by nonlinear regression as described in *Materials and Methods* and shown in Figs. 2–6. Kinetic parameters of C3b,Bb; CVF^n,Bb; and CVF^h,Bb have been reported from Refs. 12 and 28. Values are mean $\pm$ SD ($n = 3$ or 4) except for those marked with an asterisk, in which $n < 3$.

[b] C3b/cell was measured by radiolabeled Bb binding described in *Materials and Methods.*

Michaelis-Menten equation, shown as the Eadie-Hofstee plot (Fig. 5, *inset*). The K_m of self-amplified C5 convertases examined on all three surfaces exhibited values in the range of 0.074–0.016 μM (Table I). Due to the low K_m, care was taken to ensure that substrate consumption was kept low during the assays by using low concentrations of enzyme. In most assays, cleavage of C5 was <1%, whereas in those at substrate concentrations below 100 nM cleavage of C5 was less than 5% at the end of the 15-min assay. The very low K_m of these C5 convertases indicates a stronger interaction with C5 that is greater by three orders of magnitude than that observed with monomeric C3/C5 convertase, C3b,Bb ($K_m = 24$ μM).

The K_m of self-amplified C5 convertases was observed to be lower than the average K_m of C5 convertases formed when all the C3b molecules on the same surface were converted to enzymes under saturating levels of factor B (Table I). These results imply that C5 convertases with different affinities for C5 exist and that the observed K_m is an average K_m. If the observed K_m was an average of a high-affinity C5 convertase ($K_m = 0.016$ to 0.074 μM, Table I)) and a low-affinity C5 convertase ($K_m = 5.0$ or 24 μM, Table I), then because the two C5 convertases differ 100-fold or more in their affinity for C5 the Eadie-Hofstee plots would be biphasic. However, this was not observed to be the case in most of the experiments that employed different densities of C3b/cell to assemble the enzyme as observed in Figs. 2*B*, 4*A*, 4*B*, 5, and 6 indicating that the observed K_m is not an average of just a high and a low-affinity C5 convertase but instead is an average of different affinities. To investigate this further we determined the kinetic properties of C5 convertases formed on zymosan bearing 84,000 C3b/cell. Because these zymosan particles do not have a very high density of C3b/cell we would expect a larger percent of C5 convertases formed at this density of C3b/cell to have a K_m ($K_m = 5.0$ or 24 μM, Table I) that is above the average K_m of 1.39 μM obtained when all the C3b molecules are converted to enzymes under saturating levels of factor B (Table I). Therefore, initial velocities for C5 cleavage were determined up to 53 μM of C5 (10.0 mg/ml), a concentration that is 38-fold above the average K_m (1.39 μM) of the enzyme (Fig. 6). Errors involved in measuring velocities at very high substrate concentrations are large. Nevertheless, within experimental error, the data obtained appeared to fit well to the theoretical curve for a homogeneous population of enzymes based on the Michaelis-Menten equation and the Eadie-Hofstee plot had no curvature to it (Fig. 6, *inset*). Modeling the data indicated that subpopulations of C5 convertases that have K_m values

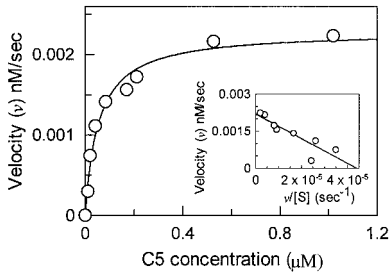

FIGURE 5. Kinetic analysis of self-amplified C5 convertases. Self-amplified C5 convertases (E_RC3b,Bb) were generated by allowing preformed C3 convertases to amplify C3b around themselves by providing excess C3. C3 convertases were preformed by incubating 50 μl of E_R at 8.0×10^9 E_R/ml (bearing 300,000 C3b/cell) with factors B (0.9 μg) and D (0.009 μg) in the presence of 1.11 mM Ni^{2+} in a final volume of 54 μl GVB buffer for 2 min at 22°C. Further formation of enzyme was stopped by the addition of 10 mM EDTA. These preformed C3 convertases were then allowed to amplify C3b around themselves for 20 min at 22°C by providing 214 μg excess C3. After amplification, the cells were washed with cold buffer to minimize loss of the remaining convertases on the cell surface and the enzymes that had not yet decayed were examined for C5 cleavage. For C5 cleavage, reactions contained saturating levels of C6 (2.5 μg, 833 nM) and the indicated concentration of C5. Reactions were initiated by adding E_SC3b,Bb to a final volume of 25 μl GVB. The assays were performed for 15 min at 37°C and the data analyzed for C5b,6 formation as described in Fig. 2.

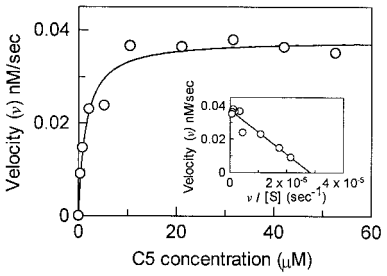

FIGURE 6. Kinetic analysis of C5 convertase activity measured over a wide range of substrate concentrations. Initial rates of C5 cleavage by C5 convertase assembled on zymosan particles bearing 84,000 C3b/cell were determined up to a concentration of 53 μM C5 as described in Fig. 2, except that ZymC3b bearing 27 ng C3b were added to the assay resulting in 6.2 nM of enzyme under saturating assay conditions. *Inset*, Analysis of the initial velocity of C5 convertase using the Eadie-Hofstee function.

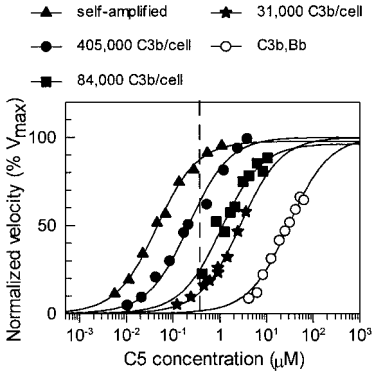

FIGURE 7. Comparison of activities of C5 convertases at the normal physiological concentration of C5 in plasma. Initial rates of C5 cleavage by ZymC3b,Bb assembled with the indicated density of C3b per cell (ranging from 31,000 to 405,000 C3b/cell) were determined by measuring the amount of C5b,6 formed at 37°C at the indicated C5 concentrations. The *y* axis represents the velocities that have been normalized by dividing by V_{max} and multiplying by 100. The vertical dashed line represents the normal plasma concentration of C5 (0.37 μM). Data for monomeric C5 convertase (C3b,Bb) is from Rawal and Pangburn (12).

10-fold or less apart would within experimental errors give linear Eadie-Hofstee plots indicating a homogeneous population of enzymes. Considered together, these results suggest that the observed K_m of C5 convertases is an average K_m of C5 convertases with varying affinities for C5.

Comparison of C5 convertase activities at the normal plasma concentration of C5

The activities of surface-bound C5 convertases assembled with different densities of C3b/cell have been compared at the normal plasma concentration of C5. Because all the three surfaces exhibited similar enzymatic properties, only data for zymosan is shown in Fig. 7. Fig. 7 shows that because C5 convertases formed with few C3b/cell have a K_m above the normal physiologic concentrations of C5 in plasma (0.37 μM; Fig. 7, vertical dashed line) these convertases will be relatively inefficient in binding C5 and, therefore, inefficient in cleaving C5. And because C5 convertases formed with a high density of C3b/cell have a K_m well below the normal plasma concentration of C5 in blood, these convertases will be occupied with C5 most of the time and the enzyme will cleave C5 at a catalytic rate close to V_{max}.

Discussion

In an attempt to enhance our understanding of the mechanism of action of natural surface-bound C5 convertases, a systematic study of C3b-dependent and cobra venom factor-dependent C5 convertases (12, 28) led us to the discovery that the simplest form of the enzyme, the bimolecular serine protease C3 convertase (C3b,Bb), was as good a C5 convertase as the more complex forms. This was evident when soluble C3 convertase (C3b,Bb) made with monomeric C3b was shown to cleave C5 without the help of a second C3b molecule and at a catalytic rate similar to that of the surface-bound enzyme formed on zymosan particles, ZymC3b,Bb, containing multiple C3b. But surface-bound C5 convertases, which are the natural forms of the enzyme, exhibit higher affinities for C5 than soluble C3b,Bb. In the present study, examination of the enzymatic properties of surface-bound C5 convertases revealed a spectrum of K_m values ranging from 5.2 to 0.016 μM depending on the density of C3b/cell (Table I). The average K_m of the enzyme was observed to decrease with increasing C3b density (Fig. 3*A*) to levels lower than the normal plasma concentration of C5 (0.37 μM; vertical dashed line in Fig. 7). These results imply that as amplification continues depositing C3b molecules on the cell surface, C5 convertases that have high affinities for C5 are generated.

The study of self-amplified C5 convertases in particular has provided new insights into the mechanism of transformation of low-affinity monomeric C3/C5 convertases to high-affinity C5 convertases. These were the highest affinity C5 convertases that were observed on cell surfaces. Preformed C3 convertases were allowed to amplify C3b around themselves by providing excess C3. After amplification, the cells were washed and the remaining initial C3 convertases were assayed for C5 cleaving properties. The K_m of these convertases for C5 (0.016–0.074 μM) (Table I) was observed to be well below the normal plasma concentration of C5 in blood (0.37 μM) suggesting that they will cleave C5 at velocities approaching V_{max} (Fig. 7) under physiological conditions. In contrast, monomeric C3/C5 convertase (C3b,Bb) because of its weak affinity for C5 (K_m = 24 μM) has been suggested to cleave 9,000 C3 for every C5 cleaved under physiological conditions (12, 28). Considered together, these results suggest that because C5 convertases formed with few C3b per cell have K_m values well above the normal concentration of C5 in blood, these convertases will be relatively inefficient in binding C5 like the monomeric C3/C5 convertase. Instead they will spend most of their time cleaving C3, thereby opsonizing the cell surface with C3b for phagocytosis. As amplification continues, C3b density increases and C3b complexes form convertases that have high affinities for C5. These convertases will predominantly cleave C5 at catalytic rates close to V_{max} (Fig. 7) thus switching the C3/C5 convertase to production of the cytolytic C5b-9 complex.

Several studies have examined the binding interaction between the substrate C5 and the noncatalytic subunit of C5 convertase and binding constants that vary by about three orders of magnitude have been reported (9, 10, 14, 28–30). The binding of C5 to C3b molecules deposited in clusters on E_S has been reported as the highest binding affinity (K_d = 0.005 μM) (9, 10), whereas that between C5 and soluble monomeric C3b has been shown to be the weakest (K_d = 37 μM) (28). Although the dissociation binding constant, K_d, and the K_m of an enzyme are not always similar, the binding affinity measured between C5 and the noncatalytic subunit of the soluble forms of C3/C5 convertases (C3b,Bb; cobra venom factor from *Naja naja kaouthia*-dependent C5 convertase; and cobra venom factor from *Naja haje haje*-dependent C5 convertase) has been shown to be nearly identical with the K_m of the respective enzyme (28). In the present study, the lowest K_m measured was for the self-amplified C5 convertase assembled on E_S (0.016 ± 0.007

μM) (Table I). The K_m is observed to be within 3-fold of the K_d (0.005 μM) reported for the binding of C5 to C3b molecules deposited in clusters on E_S (9, 10). These observations suggest that surface-bound C5 convertases like soluble C5 convertases may have similar K_d and K_m. The studies of Hong et al. (16) and Kinoshita et al. (10) have suggested the formation of C4b-C3b and C3b-C3b dimers as high-affinity C5 binding sites within the C3b clusters deposited on E_S. The additional C3b has been shown to be covalently bound to the C3b (10) or C4b (9, 18) subunit of the C3 convertase resulting in structures C3b,C4b,C2a and $C3b_2$,Bb, considered to be C5 convertases of the classical and the alternative pathway, respectively (9, 10, 17, 18). The covalent C3b-binding site has been identified as a serine residue at position 1217 on the α'-chain of C4b (9). Although no evidence is provided in the present study for the structure of high-affinity C5 convertases, we attribute the low K_m of the enzyme to the formation of C3b-C3b complexes.

The functional role of C3b dimers has been suggested to be able to hold the substrate C5 in a rigid conformation so as to enable cleavage by Bb. Hong et al. (16) reconstituted C5 convertase activity with purified C3b dimers or oligomers in the presence of Ni^{2+} and factors B and D. Their studies showed higher C5 convertase activity with C3b oligomers and dimers than with monomeric C3b. Studies by Jelezarova et al. (31) have suggested C3b-C3b dimers in complex with IgG molecules to form better precursors of convertases than C3b. Recent studies by Sandoval et al. (32) involving indels have identified at least two binding sites on C5 for the classical pathway C5 convertase in addition to the convertase cleavage site. Based on all these studies it appears that C5 binds with high affinity to multimeric C3b complexes through interactions of at least two distinct sites on C5 and at least two sites on C3b.

In contrast to the K_m of surface-bound C5 convertases, which varied by approximately three orders of magnitude (Fig. 3*A* and Table I), the k_{cat} of the enzyme varied very little (Fig. 3*B*). C5 convertases assembled on zymosan particles exhibited similar catalytic rates at all densities of C3b/cell. Those formed on E_R and E_S at low density of C3b/cell exhibited k_{cat} that was similar to that of the soluble monomeric C3b,Bb but showed a 3- to 4-fold decrease with increasing density of C3b/cell (Table I). Although this decrease in k_{cat} was consistently observed with increasing density of C3b/cell, its origin is not apparent. High densities of C3b,Bb, or bound C5 could interfere with efficient C5 cleavage or restrict access to neighboring active sites. The k_{cat} of the self-amplified C5 convertases might be revealing in this regard, but the k_{cat} of these enzymes was impossible to measure accurately due to the low numbers of enzymes per cell and to enzyme decay before and during the assays (these enzymes were premade and no factor B was available for enzyme reformation as in other kinetic assays). Nevertheless, the average k_{cat} for cell-bound alternative pathway C5 convertases was 0.0056 s^{-1} indicating that the rate of C5 cleavage is one of the slowest enzymatic reactions known with one C5 cleaved per 3 min at V_{max}. Apparently this level of activity is sufficient to generate biologically effective levels of cytolytic C5b-9 and the chemotactic fragment C5a.

The proposed mechanism underlying the conversion of low affinity to high-affinity C5 convertases is schematically represented in Fig. 8. The first C3b molecule deposited on the surface, forms a monomeric C3/C5 convertase with factor B. This C3/C5 convertase, because of its weak affinity for C5, will function primarily as a C3 convertase cleaving thousands of C3 for each C5 cleaved (12), thereby amplifying C3b around the first C3/C5 convertase. As C3b deposition occurs, C3b complexes are formed that have higher affinities for C5. This results in the conversion of low-affinity C5 convertase to high-affinity C5 convertase. At physiological C5 concentrations these enzymes exceed 90% saturation with C5, which prevents C3 cleavage and allows C5 cleavage at a velocity approaching V_{max}. The C3b molecules at the edges of the expanding amplification ring will be mostly monomers and will form monomeric C3/C5 convertase. The monomeric C3/C5 convertases will spend most of their time cleaving C3 because of their weak affinity for C5. As amplification continues outward, deposition of C3b converts this region into high-affinity C5 convertases and produces another ring of monomers. This process continues until the surface is completely coated with C3b and most C3/C5 convertases have been converted to high-affinity C5 convertases. At this point, net C3b deposition ceases and a plateau in C3b per cell is reached (33).

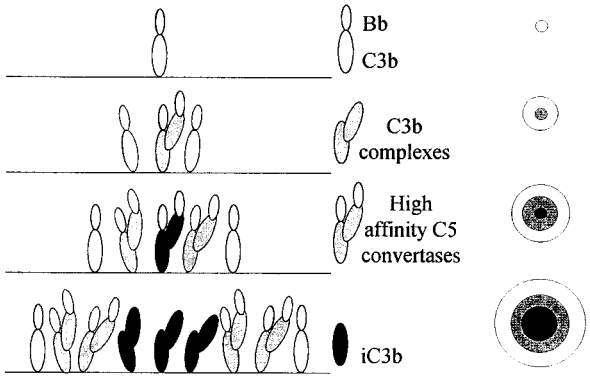

FIGURE 8. Model for formation and inactivation of high-affinity C3/C5 convertases during alternative pathway activation. Both side views (*left*) and top views (*right*) of the activating surface are shown. The first C3b molecule deposited on the target surface forms a monomeric C3/C5 convertase C3b,Bb. C3b,Bb cleaves C3, depositing C3b on and near itself. Some of the newly deposited C3b molecules will form C3b-C3b complexes, which have higher affinities for C5, thereby switching the enzyme from cleavage of C3 to cleavage of C5. These convertases will predominantly cleave C5 at a velocity close to V_{max} resulting in higher production of the cytolytic C5b-9 complex. The C3b molecules on the outer edge of the expanding ring of amplification have low affinities for C5. Because these convertases will interact weakly with C5, they will predominantly cleave C3, amplifying C3b on the surface. As amplification continues expanding outward, additional C3b molecules are deposited and the process of creating new high-affinity C5 convertases continues. This results in a mixed population of C5 convertases with varying affinities for C5 and accounts for the average K_m of the enzyme observed in the present study (Table I) when all the C3b molecules are converted to convertases. Under physiological conditions, the initial C5 convertases, located in the center of the expanding ring, will be subjected to inactivation by factors H and I and these convertases will stop production of both C3b and C5b-9. In this way the sequential waves of amplification, formation of high-affinity C5 convertases and their inactivation continues outward from the initial C3b resulting in both opsonization of the cell surface with C3b/iC3b/C3d for phagocytosis and to production of the cytolytic C5b-9 complex.

Although, in the present study the effect of the complementary protein factors H and I was not examined, it is informative to envisage their functional roles in inactivating high-affinity C5 convertases during complement activation. The higher affinity C5 convertases formed in the center of the expanding ring of amplification will be inactivated by the decay-accelerating activity of factor H (decay of Bb) and the cofactor activities of factors H and I. The effect of these regulatory proteins would be the conversion of C3b to iC3b with the eventual formation of C3d. This process of C3 cleavage, C3b amplification, formation of high-affinity C5 convertases, C5a release, C5b-9 formation, and C3b inactivation are all critical for opsonization, phagocytosis, cytolysis, and presentation

of the cell surface of the invading microorganism to the adaptive immune system through receptors CD21/CD19.

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References

1. Reid, K. B. M. 1998. Clasical pathway of activation. In *The Complement System*. 2nd Ed. K. Rother, G. O. Till, and G. M. Hänsch, eds. Springer, New York, p. 68.
2. Reid, K. B. M. 1998. Lectin pathway of nonself recognition. In *The Complement System*. 2nd Ed. K. Rother, G. O. Till, and G. M. Hänsch, eds. Springer, New York, p. 86.
3. Pangburn, M. K. 1998. Alternative pathway: activation and regulation. In *The Complement System*. 2nd Ed. K. Rother, G. O. Till, and G. M. Hänsch, eds. Springer, New York, p. 93.
4. Ember, J. A., M. A. Jagels, and T. E. Hugli. 1998. Characterization of complement anaphylatoxins and their biological responses. In *The Human Complement System in Health and Disease*. J. E. Volanakis and M. M. Frank, eds. Dekker, New York, p. 241.
5. Wetsel, R. A. 1990. Structure, function and cellular expression of complement anaphylatoxin receptors. *Curr. Opin. Immunol. 7:48.*
6. Daha, M. R., D. T. Fearon, and K. F. Austen. 1976. C3 requirements for formation of alternative pathway C5 convertase. *J. Immunol. 117:630.*
7. Medicus, R. G., R. D. Schreiber, O. Götze, and H. J. Müller-Eberhard. 1976. A molecular concept of the properdin pathway. *Proc. Natl. Acad. Sci. USA 73:612.*
8. Medicus, R. G., O. Götze, and H. J. Müller-Eberhard. 1976. Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway. *J. Exp. Med. 144:1076.*
9. Takata, Y., T. Kinoshita, H. Kozono, J. Takeda, E. Tanaka, K. Hong, and K. Inoue. 1987. Covalent association of C3.b with C4b within C5 convertase of the classical complement pathway. *J. Exp. Med. 165:1494*.
10. Kinoshita, T., Y. Takata, H. Kozono, J. Takeda, K. Hong, and K. Inoue. 1988. C5 convertase of the alternative complement pathway: covalent linkage between two C3b molecules within the trimolecular complex enzyme. *J. Immunol. 141:3895*.
11. Masaki, T., M. Matsumoto, R. Yasuda, R. P. Levine, H. Kitamura, and T. Seya. 1991. A covalent dimer of complement C4.b serves as a subunit of a novel C5 convertase that involves no C3 derivatives. *J. Immunol. 147:927*.
12. Rawal, N., and M. K. Pangburn. 1998. C5 convertase of the alternative pathway of complement: kinetic analysis of the free and surface-bound forms of the enzyme. *J. Biol. Chem. 273:16828.*
13. Vogt, W., G. Schmidt, B. Von Buttlar, and L. Dieminger. 1978. A new function of the activated third component of complement: binding to C5, an essential step for C5 activation. *Immunology 34:29.*
14. Isenman, D. E., E. R. Podack, and N. R. Cooper. 1980. The interaction of C5 with C3b in free solution: a sufficient condition for cleavage by fluid phase C3/C5 convertase. *J. Immunol. 124:326.*
15. Dias, D. A., W. Silva, and I. H. Lepow. 1967. Complement a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified components of human complement. *J. Exp. Med. 125:921.*
16. Hong, K., T. Kinoshita, P. Pramoonjago, Y. U. Kim, T. Seya, and K. Inoue. 1991. Reconstitution of C5 convertase of the alternative complement pathway with isolated C3b dimer and factors B and D. *J. Immunol. 146:1868*.
17. Kozono, H., T. Kinoshita, Y. U. Kim, Y. Takata-Kozono, S. Tsunasawa, F. Sakiyama, J. Takeda, K. Hong, and K. Inoue. 1990. Localization of the covalent C3b-binding site on C4b within the complement classical pathway C5 convertase, C4b2a3b. *J. Biol. Chem. 265:14444*.
18. Kim, Y. U., M. C. Carroll, D. E. Isenman, M. Nonaka, P. Pramoonjago, J. Takeda, K. Inoue, and T. Kinoshita. 1992. Covalent binding of C3.b to C4b within the classical complement pathway C5 convertase: determination of amino acid residues involved in ester linkage formation. *J. Biol. Chem. 267:4171.*
19. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large scale isolation of functionally active components of the human complement system. J. Biol. Chem. *256:3995.*
20. Pangburn, M. K. 1987. A fluorimetric assay for native C3: the hemolytically active form of the third component of human complement. *J. Immunol. Methods 102:7.*
21. Götze, O., and H. J. Müller-Eberhard. 1971. The C3-activator system: an alternative pathway of complement activation. *J. Exp. Med. 134:90s.*
22. Lesavre, P. H., T. E. Hugli, A. F. Esser, and H. J. Müller-Eberhard. 1979. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol. 123:529.*
23. Kolb, W. P., L. M. Kolb, and J. R. Savar. 1982. Biochemical characterization of the sixth component (C6) of human complement. *Biochemstry 21:294.*
24. Tack, B. F., and J. W. Prahl. 1976. Third component of human complement: purification from plasma and physicochemical characteriztion. *Biochemistry 15: 4513.*
25. Sahu, A., T. R. Kozel, and M. K. Pangburn. 1994. Specificity of the thioester-containing site of human C3 and its significance to complement activation. *Biochem. J. 302:429.*
26. Pangburn, M. K., and H. J. Müller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of BIH control and generation of restriction on neuraminidase treated cells. *Proc. Natl. Acad. Sci. USA 75:2416.*
27. Christopherson, R. I. 1983. Desalting protein solutions in a centrifuge column. *Methods Enzymol. 91:278.*
28. Rawal, N., and M. K. Pangburn. 2000. Functional role of the noncatalytic subunit of complement C5 convertase. *J. Immunol. 164:1379.*
29. Discipio, R. G. 1981. The conversion of human complement component C5 into fragment C5b by the alternative pathway C5 convertase. *Biochem. J. 199:497.*
30. Discipio, R. G. 1981. The binding of human complement proteins C5, factor B, BIH and properdin to complement fragment C3b on zymosan. *Biochem. J. 199: 485.*
31. Jelezarova, E., and H. U. Lutz. 1999. Assembly and regulation of the complement amplification loop in blood: the role of C3b-C3b-IgG complexes. *Mol. Immunol. 36:837.*
32. Sandoval, A., R. Ai, J. M. Ostresh, and R. T. Ogata. 2000. Distal recognition site for classical pathway convertase located in the C345C/Netrin module of complement C5. *J. Immunol. 165:1066.*
33. Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard. 1983. C3b deposition during activation of the alternative complement pathway and the effect of deposition of the activating surface. *J. Immunol. 131:1930.*