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Third Component of Complement (C3): Structural Properties in Relation to Functions

(topology of functional sites/physiologic C3 fragments/C3-membrane interaction/ immune adherence/enzyme subunit)

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ABSTRACT **The third component of complement (C3) fulfills a pivotal role in the functions of the complement system. We have investigated the topological relationships among its polypeptide chains, physiologic fragments, enzyme attack regions, and functional sites. C3 consists of two chains (α and β) which are linked by disulfide bonds and noncovalent forces and which have molecular weights of, respectively, 120,000 and 75,000. C3 is activated by action of C3 convertase on the α-chain. With hydrolysis of one polypeptide bond, C3a, the 9000 dalton activation peptide is dislocated from the NH_2-terminal portion of the α-chain. A previously concealed binding region is thereby transiently revealed in the C3b-fragment (181,000 dalton) which displays affinity for apparently nonspecific acceptors present on biological membranes. Binding of nascent C3b to membranes occurs through the C3d portion of the fragment because subsequent action of the C3b-inactivator or trypsin on bound C3b causes release of C3c, but not of C3d. Bound C3b and C3d possess stable sites that are capable of binding to specific receptors present on a limited variety of cells. We propose that all known physiologically occurring fragments of C3 arise by enzymatic cleavage of the α-chain: C3a, C3b, C3c, and C3d. Whereas C3a (α_1) and C3d (α_2) consist of a single chain and C3b consists of two chains (α' and β), C3c is composed of the entire β-chain and multiple fragments of the α-chain, each of which is linked by disulfide bonds to the β-chain.**

The aim of this communication is to describe the topological relationships among chains, fragments, functional sites, and enzymatic attack regions of the C3 molecule (third component of complement).

C3, which was first recognized and isolated in 1960 (1), fulfills multiple physiological functions in host defense against pathogenic microorganisms and probably transformed host cells. It occurs in plasma and in other body fluids in inactive, but activatable form. The activating enzyme, C3 convertase or $C\overline{4,2}$, cleaves the molecule into two fragments, C3a and C3b (2, 3). The activation peptide, C3a, constitutes one of the two known anaphylatoxins. In very low concentrations the peptide effects release of histamine from mast cells (4), chemotactic migration of polymorphonuclear leukocytes (3, 5), and contraction of smooth muscle (2, 3, 6). *In vivo* it is phlogogenic and causes formation of cutaneous edema and erythema (7, 8).

The large fragment, C3b, in its nascent state, is capable of binding to the surface of cells (9) and other particles, including bacteria and immune complexes and to facilitate their ingestion by phagocytic cells (10). It mediates the immune adherence reaction. C3b also functions as subunit or modulator of several complex enzymes of the classical and alternative complement pathway (11, 12). Native C3 is operative as the hydrazine-sensitive factor of the properdin system (12). In addition, C3, and especially C3b, have been implicated as mediators in the stimulation of antibody-forming cells (13, 14).

The functions of C3a and C3b are controlled, respectively, by the serum enzymes carboxypeptidase B (or anaphylatoxin inactivator) (15) and C3b-inactivator (16, 17). Serum carboxypeptidase B removes the COOH-terminal arginyl residue from C3a (18), and C3b-inactivator cleaves C3b into the fragments C3c and C3d (19, 20).

The biological significance of C3 in man has been documented by the observation of recurrent severe bacterial infections in a patient with inherited homozygous C3 deficiency (21) and in a patient with congenital C3 hypercatabolism (22) due to a genetic deficiency of the C3b-inactivator (23).

MATERIALS AND METHODS

C3 was isolated as described (1). C3a was produced and isolated according to Hugli *et al.* (24). C3b was obtained by treatment of C3 with either C3 convertase (25) or trypsin (3). C3c and C3d were prepared by treatment of C3b with trypsin (3), or these fragments were directly isolated from serum. For this purpose fresh human serum containing 0.05% NaN_3 was incubated for 5 days at 37°, under which conditions C3 is degraded and these fragments accumulate. Alternatively, 250 ml of fresh human serum was incubated at 37° for 1 hr with 2 mg of isolated cobra venom factor (26) and was subsequently held at 4° overnight. C3c and C3d were isolated by a three-step procedure. Treated serum (250 ml) was dialyzed against phosphate buffer, pH 8.1, having a conductance of 4 mmho/cm. The serum was applied to a 3-liter column of DE32 cellulose equilibrated with the same buffer. The fragments were eluted with a NaCl concentration gradient; elution of C3c and C3d occurred at 7 and 13 mmho/cm, respectively. The fragments were detected in the eluate by immunochemical techniques using monospecific antisera to each fragment. The C3c pool was applied to a hydroxyapatite column (300 ml) equilibrated with a sodium-potassium phosphate buffer, pH 8, and a conductance of 8 mmho/cm. The column was washed with 500 ml of the same buffer and 500 ml of sodium-potassium phosphate buffer, pH 8, and a conductance of 12 mmho/cm. The fragment was eluted with buffer having a conductance of 16 mmho/cm. The eluted material was concentrated to 8 ml and subjected to Pevikon block electrophoresis using barbital buffer, pH 8.6, T/2 = 0.05, 3 v/cm, and 48 hr. The C3d pool from the DE32 cellulose column was concentrated to 15 ml and applied to a 4-liter Sephadex G-200 column equili-

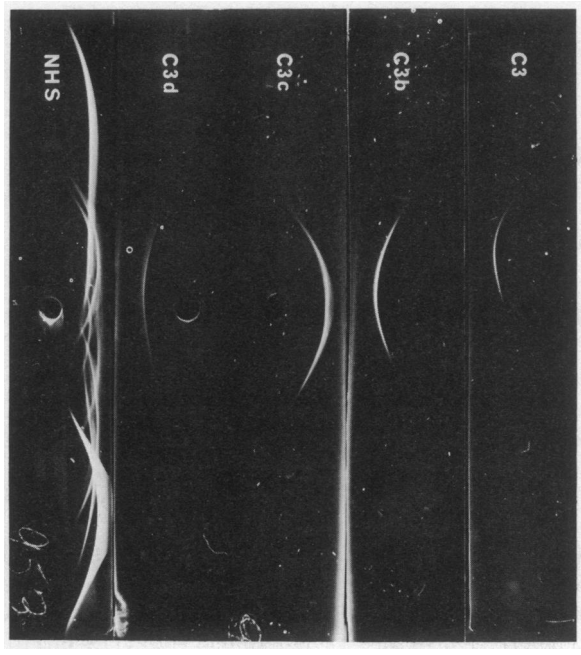

FIG. 1. Immunoelectrophoretic representation of isolated human C3 and three of its physiological fragments: C3b, C3c, and C3d. The figure represents a composite of several different photographic records. The patterns were developed with an antiserum to native C3. For comparison, the pattern of whole human serum (NHS) is shown, which was developed with a corresponding antiserum.

brated with 0.1 M sodium phosphate buffer, pH 7.2. The C3d pool was concentrated to 8 ml and subjected to Pevikon block electrophoresis as described above. The total yield of C3c was 8–10 mg and of C3d 1–2 mg. The purity of C3 and its fragments was evaluated by polyacrylamide gel electrophoresis in alkaline buffer (C3, C3b, C3c, and C3d) and in acid buffer (C3a). Molecular weights of reduced and unreduced C3 and C3 fragments were determined according to the method of Weber and Osborn (27) as modified by Mosesson (28). The following reference substances of known molecular weight were used: β-galactosidase (130,000), phosphorylase *a* (94,000), fibrinogen chains Aα (68,000), Bβ (56,000), and γ (48,000), glucose-6-phosphate dehydrogenase (36,000), and chymotrypsinogen (25,700). Disc electrophoresis was done according to Davis (29), and immunoelectrophoresis by the standard procedure in agar gels at pH 8.6.

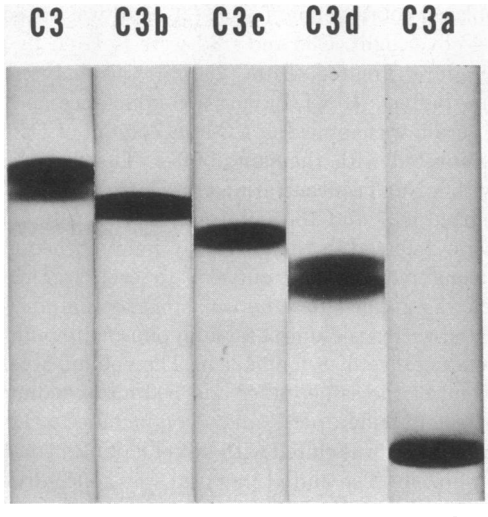

FIG. 2. Disc gel electrophoresis of C3 and its physiological fragments. The four gels on the left were electrophoresed at alkaline pH with the cathode at the top. The gel on the right was electrophoresed at acid pH with the cathode at the bottom.

Sheep erythrocytes (E) sensitized with antibody (A) to E (EA) were treated with C1, C4, and oxidized C2 (^{oxy}C2) to form stable EAC1$\overline{4,2}$, as described (30). This intermediate complex was then converted to EAC$\overline{4,2}$,3b, as described, using ^{125}I-labeled C3 (9). These cells were treated at 5×10^8 per ml with 2.5–25 μg of trypsin per ml. The cells were suspended in isotonic veronal buffer, pH 7.3, containing 0.1% human serum albumin for 10 min at 20°. Five to 50 μg of soybean trypsin inhibitor was added and allowed to react with trypsin for 2 min at 20°. The cells were washed three times with 3 ml veronal/serum albumin, and the pellet was suspended in 100 μl of veronal/serum albumin and held at 4° overnight. Twenty-five microliters of the supernatant was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis in reduced (1 mM dithiothreitol) or unreduced form. In other experiments 1–10% (v/v) of fetal calf serum was used instead of trypsin. After 10 min at 20° the cells were washed and radioactive material was eluted and analyzed as described above. After electrophoresis the gels were sliced into 20 segments, and the radioactivity was quantitated in an automatic gamma counter.

Immune adherence was carried out as described and hemagglutination was performed with antisera raised in rabbits to the isolated fragments C3c and C3d.

RESULTS

Isolated C3 and its enzymatic degradation products, C3b, c, and d, which are immunochemically defined, are demonstrated by immunoelectrophoresis in Fig. 1. The same preparations were analyzed by alkaline polyacrylamide gel electrophoresis as shown in Fig. 2, which includes C3a analyzed by acid polyacrylamide gel electrophoresis. Doubling of the protein zone by this technique has at times been observed for C3 and the fragment C3d, but not for C3a, C3b, or C3c.

The subunit composition of C3, C3b, c, and d is demonstrated in Fig. 3. Upon reduction in the presence of sodium dodecyl sulfate, C3 is dissociated into two polypeptide chains, α and β, having molecular weights of 120,000 and 75,000, respectively. Identical treatment of C3b resulted in dissociation of the fragment into two chains, α′ (111,000 dalton), which is by 9000 daltons smaller than the α-chain of the native molecule, and the β-chain (75,000 dalton). The reduction of the molecular weight of the α-chain corresponds to enzymatic removal of C3a during conversion of C3 to C3b.

C3c has a molecular weight of 140,000, and upon sodium dodecyl sulfate gel electrophoresis after reduction yields a pattern consisting of several bands (Fig. 3). The major band corresponds to a polypeptide chain with a molecular weight identical to that of the β-chain. Two minor bands represent fragments with molecular weights of 25,000 and 20,000. The band corresponding to the 25,000 dalton material at times exhibited separation into two similar bands and was consistently more heavily stained than the 20,000 dalton fragment, suggesting that the 25,000 dalton material consists of two fragments of similar size.

The molecular weight of C3d is 25,000 and was unchanged

TABLE 1. *Assigned molecular weights of C3, C3 fragments, polypeptide chains, and polypeptide chain fragments*

C3, C3 fragments	Chains, chain fragments	Molecular weights	Sum of molecular weights
C3	$\alpha + \beta$	190,000	
C3a	α_1	9,000	190,000
C3b	$\alpha' + \beta$	181,000	
C3c	$\alpha_4 + \beta$	140,000	165,000
C3d	α_2	25,000	
C3	α	120,000	195,000
	β	75,000	
α-Chain of C3	α_1	9,000	
	α_2	25,000	120,000
	α_3	16,000	
	α_4	70,000	
α-Chain portion of C3c	α_{4I}	25,000	
	α_{4II}	20,000	70,000
	α_{4III}	25,000	

after reduction in sodium dodecyl sulfate, indicating that it represents a fragment of a single chain. The molecular weight values of C3, C3 fragments, chains, and chain fragments are listed in Table 1. The subunit composition of the fragments was independent of the method used for their production: C3b was composed of α'- and β-chain regardless of whether obtained from C3 by trypsin or C3 convertase. C3c and C3d showed identical patterns when these fragments were isolated from aged serum or from serum treated with cobra venom, or produced from C3b by trypsin treatment.

The following experiments were performed to determine the mode of binding of C3b to cells and the effect of proteases on the structure of cell-bound C3b. Sheep erythrocytes (EA) with bound $C\overline{4,2}$-enzyme containing C2 in oxidized form, were treated with ^{125}I-labeled C3. The resulting EA$C\overline{4,2}$,3b cells carried an average of 12,000 C3b molecules per cell. These cells were positive in the immune adherence reaction and could be agglutinated with antiserum to either C3c or C3d. Cells were treated with proteases for 10 min at 20° and was followed by washing and overnight elution of radioactive material at 4°. The composition of the eluted material, as revealed by dodecyl sulfate/polyacrylamide gel electrophoresis with and without prior reduction, is shown in Fig. 4. Buffer eluates of EA$C\overline{4,2}$,3b contained only material of the size and chain structure of C3b. Trypsin or fetal calf serum eluted two molecular species, one corresponding to C3b and the other having the size of C3c. The latter, upon reduction, dissociated into 75,000 and 25,000 dalton pieces. This composition is similar to reduced C3c shown in Fig. 3.

Trypsin- or serum-treated EA$C\overline{4,2}$,3b retained agglutinability with anti-C3d but showed diminished reactivity with anti-C3c, which was marked at the highest dose of enzyme used. Similarly, immune adherence was reduced in proportion to the amount of enzyme used.

DISCUSSION

C3 is composed of two distinct chains which are held together by disulfide bonds and noncovalent forces. This observation confirms an earlier report by Nilsson and Mapes (31). The native C3 molecule possesses two binding sites for specific receptors that are present on a variety of mammalian cells

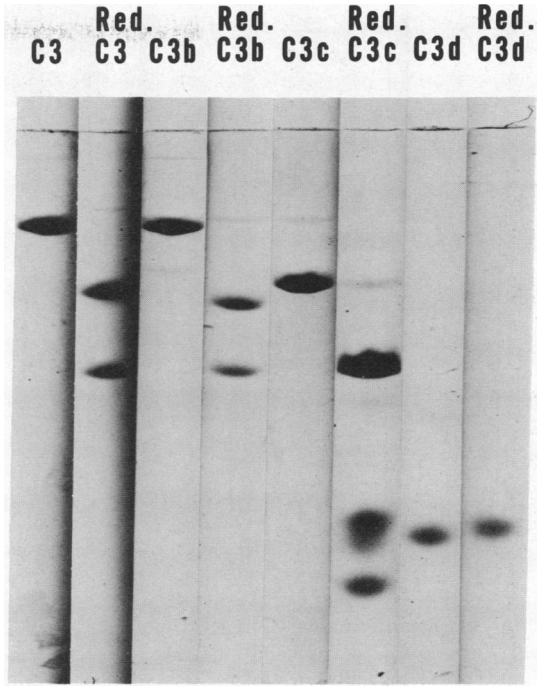

FIG. 3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of reduced and unreduced C3, C3b, C3c, and C3d.

and are referred to as the C3b- and C3d-receptors. The binding of native, monomeric C3 to cells with either C3b- or C3d-receptors has been shown, using radiolabeled C3 or fluoresceinated antibody to C3 (32). The molecule is endowed with

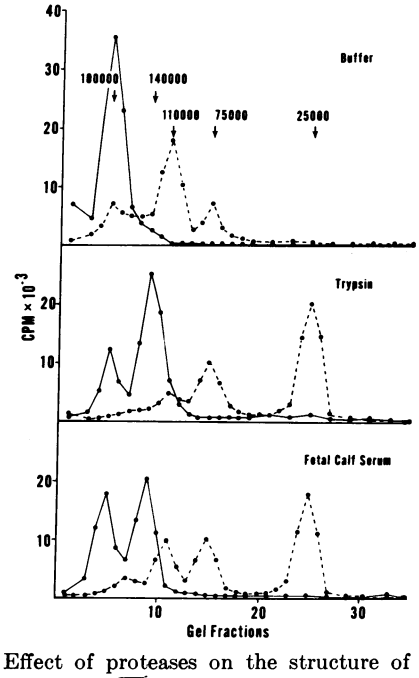

FIG. 4. Effect of proteases on the structure of cell-bound, radiolabeled C3b. EA$C\overline{4,2}$,3b was treated with trypsin, fetal calf serum, or buffer for 10 min at 20°, which was followed by washing and overnight elution of radioactive material at 4°. The eluted material, in reduced (*dashed line*) and unreduced (*solid line*) form, was analyzed by dodecyl sulfate/polyacrylamide gel electrophoresis.

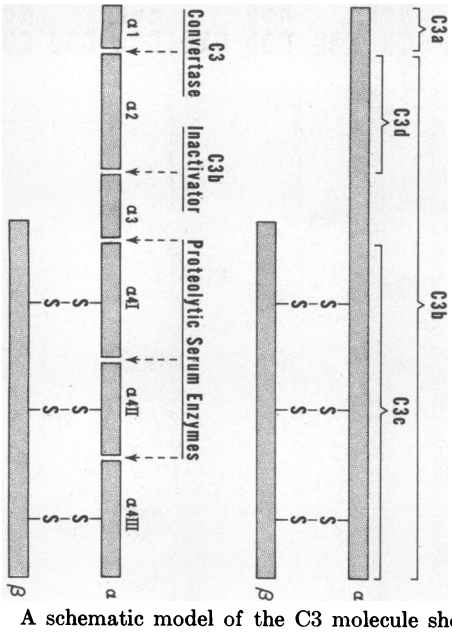

FIG. 5. A schematic model of the C3 molecule showing the proposed topological relationship between the polypeptide chains and physiologically occurring, enzymatically produced fragments.

a concealed binding site for nonspecific receptors that is exposed by enzymatic cleavage of C3 (9). The two stable and specific binding sites of C3 are retained by C3b (33). More extensive proteolysis of C3b gives rise to the fragments C3c and C3d. C3 is antigenically distinct from its fragments by at least one determinant, called B (19). Complete antigenic nonidentity exists between the a- and the b-fragments and the a-, c-, and d-fragments (3).

Attack of C3 by C3 convertase has the following immediate effects: (*i*) the liberation of C3a anaphylatoxin, (*ii*) the generation of a labile, nonspecific binding site on C3b, (*iii*) expression of immune adherence activity by bound C3b, (*iv*) modulation by C3b of two complement enzymes, and (*v*) acquisition by C3b of susceptibility to degradation by the enzyme C3b-inactivator and other serum enzymes.

The first effect is caused by hydrolysis of probably a single peptide bond located between residues 77 and 78 (34) of the α-chain. C3a has been assigned, in the proposed model (Fig. 5), the position of the NH_2-terminal portion of the α-chain because (*i*) the α-chain is smaller in C3b than in C3, (*ii*) C3a consists of a single chain (34), (*iii*) both C3a and C3 have serine at the amino terminus (18), and (*iv*) C3a possesses a detectable carboxy-terminal residue (arginine), whereas the precursor molecule, C3, does not (18). C3a represents an unusual portion of the intact C3 molecule. It possesses a high degree of secondary structure, a disulfide knot involving six half-cystine residues, and a pronounced positive charge (34, 35). The α-helical structure, the disulfide arrangement, and the COOH-terminal arginine are essential for expression of its anaphylatoxin activity (15, 35). This activity depends on a binding site in C3a for cellular receptors that is independent of the COOH-terminal arginyl residue, and on an active region that depends on the COOH-terminal residue (4).

The second effect, also caused by C3 convertase, results in the exposure of a labile binding site in nascent C3b, through which it can attach itself to the surface of cell membranes, immune complexes, particulate lipopolysaccharides, and other particles. This site appears to be located in the d-portion of C3b for the following reasons: (*i*) When EAC$\overline{4,2}$,3b is treated with proteases, C3c is released while C3d remains cell-bound. (*ii*) Upon binding of C3b to particles, the stable site that is specific for C3d receptors is largely concealed, whereas it is reactive on soluble C3b (33). The labile site has a half-life of milliseconds, and upon collision with particles, C3b becomes firmly bound. The chemical nature of the receptor is unknown; however, it is a common constituent of biological membranes, because nascent C3b can be bound to all cells tested to date.

One of the consequences of binding of C3b through its labile site to particles is their ability to adhere to C3b-receptor bearing cells through the stable binding site on bound C3b. This mechanism underlies the immune adherence phenomenon and is probably responsible for complement-dependent enhancement of phagocytosis.

The fourth effect is the appearance of groups on C3b that allow it to function as a modulator of the C$\overline{4,2}$ enzyme and of the C3 proactivator–C3 proactivator convertase system. Association of C3b with the C$\overline{4,2}$ enzyme provides a substrate binding site with specificity for C5 (11). In the alternative or properdin pathway, C3b constitutes a subunit of the C3-cleaving enzymes (12).

All known functions of C3b are abolished by the action of the C3b-inactivator. This serum enzyme cleaves C3b into the c- and d-fragments. Cleavage appears to occur exclusively on the α-chain, probably affecting a bond linking the α_2 and α_3 segments of the chain (Fig. 5). This site of cleavage is suggested by the observation that on cleavage of particle-bound C3b, C3d or α_2 remains firmly attached while C3c is released. It is this fact that indicates that the labile binding site is located in the d-portion of C3b. After dislocation or dissociation of C3c, C3d displays a stable binding site that is specific for C3d-receptors on B-lymphocytes and monocytes. Unlike the C3b-receptor, which is operative in the phagocytic reaction, the biological function of the C3d-receptor is presently uncertain.

In addition to the α_2–α_3 cleavage of the C3b-inactivator, at least two other bonds are split in C3c by unidentified serum enzymes, as evidenced by the appearance upon reduction of C3c of at least three fragments. Since the molecular weight of the large peptide of reduced C3c is identical to that of the β-chain, this peptide most probably represents the unaltered β-chain. By implication, the additional fragments arose by cleavage of the α-chain segment of C3c, as indicated in the model shown in Fig. 5. Since the molecular weight of C3c is 140,000, and the sum of the molecular weights of the fragments of reduced C3c is 120,000, a fragment of approximately 20,000 dalton is unaccounted for. However, doubling of the 25,000 dalton band of the polyacrylamide gel pattern suggests that the missing fragment is part of this material. Therefore, C3c may consist of four fragments held together by disulfide bridges.

The existence of one additional fragment (α_3, molecular weight 16,000) of the α-chain is suggested by the difference of the molecular weight of C3 and the sum of the molecular weights of C3a, C3c, and C3d. This fragment is proposed to be a part of C3b which is liberated subsequent to C3b-inactivator action. It has not been observed as a distinct entity in these investigations; however, others have reported the occurrence of an 18,000 dalton peptide with leukocyte mobilization activity upon activation and degradation of C3 in whole

serum (36). The possibility is raised that the leukocyte mobilization factor is related to the α_3-fragment.

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