MEMBRANE ATTACK COMPLEX OF COMPLEMENT

A Structural Analysis of Its Assembly*

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The membrane attack complex (MAC)[1] of complement has been shown to be a dimer of C5b-9 (1) and the ring-shaped ultrastructural membrane lesion induced by complement (2) was identified as the membrane-bound MAC (1, 3). Electron microscopic examination of the isolated MAC has revealed a 50- × 200-Å ring structure with 100-Å-long perpendicular attachments. The latter structures may represent stalk-like appendages (1) or the wall of a hollow cylinder (3). Although the precise geometry of the MAC remains uncertain at present, it appeared feasible to gain insight into the process of MAC assembly by a comparative study of the morphology and particle size of the intermediate complexes C5b-6, C5b-7, C5b-8, and C5b-9. For this purpose recombinants of the intermediate complexes and phospholipids were prepared using dioleoyl lecithin single bilayer vesicles and purified human C5b-6, C7, C8, and C9. The results show that each intermediate complex has a characteristic morphology which is referable to the overall morphology of the fully assembled MAC and that MAC assembly constitutes an intricate process involving both protein association and dissociation reactions. It is proposed that production of a functional membrane lesion by the MAC is the result of phospholipid reorganization caused by highly complex protein-protein and protein-phospholipid interactions.

Materials and Methods

Chemicals. Dioleoyl lecithin (DOL) was purchased from Avanti Biochemicals, Inc. (Birmingham, Ala.), sodium deoxycholate (DOC) was obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of the best grade commercially available.

Complement Proteins. C5b-6 (4), C7 (5), C8 (6), and C9 (7, 8) were purified according to published procedures. The proteins were radiolabeled by the method of McConahey and Dixon (9).

Crystallization of C5b-6. C5b-6 at concentrations between 1 and 2.5 mg/ml dissolved in veronal (3.3 mM)-buffered saline (0.15 M), pH 7.4, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (VB) was held at 4°C for 72 h. The resulting paracrystals were sedimented in a table-

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[1] *Abbreviations used in this paper:* DOC, deoxycholate; DOL, dioleoyl lecithin; MAC, membrane attack complex(es); PTA, sodium phosphotungstate; VB, veronal (3.3 mM)-buffered saline (0.15 M), pH 7.4, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

top centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) and resuspended in VB.

Preparation of C5b-7 Protein Micelles. 75 μg ^{125}I-C5b-6 (0.5 μCi) and 30 μg ^{131}I-C7 (0.1 μCi) were mixed in a total vol of 0.1 ml VB and incubated for 10 min at 30°C. C5b-7 micelles were separated from unreacted material by sucrose density-gradient ultracentrifugation in 5 ml linear, 10–50% sucrose density gradients (prepared in VB) for 16 h at 36,000 rpm in a SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation, eight-drop fractions were collected from the top by means of a Buchler Densi Flow IIc Collector (Buchler Instruments Div., Searle Diagnostics Inc., Fort Lee, N. J.) and analyzed for radioactivity. ^{125}I-SC5b-9 (23S) and ^{125}I-MAC (33.5S) served as reference proteins. Peak fractions containing radioactivity of both ^{125}I-C5b-6 and ^{131}I-C7 and sedimenting at 36S were pooled, and concentrated to 200 μl and simultaneously freed of sucrose by electrophoresis in a model 1750 Electrophoretic Sample Concentrator (ISCO [Instrumentation Specialties Co.], Lincoln, Nebr.). The concentrated samples were examined by electron microscopy as described below.

Preparation of DOL-Intermediate Complexes. DOL-C5b-7 vesicles: these were prepared by a modification of the gel filtration procedure of Brunner et al. (10). 5 mg DOL was dried in vacuo and the dry residue dispersed in 0.5 ml buffer, pH 8.1, containing 0.09 M NaCl, 0.02 M Tris-acetate, 12 mM DOC, 2 mM EDTA, and 0.02% NaN_3. 190 μg C5b-6 or ^{125}I-C5b-6 (1 μCi) was added to 0.2 ml of clear solution of DOL-DOC mixed micelles. After 1 min at 37°C, 55 μg ^{131}I-C7 or ^{125}I-C7 (1 μCi) was added and the incubation continued for 10 min at 37°C. The sample was then applied to a 1.5- × 30-cm column of Sephadex G50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) equilibrated with buffer, pH 8.1, containing 0.09 M NaCl, 0.02 M Tris-acetate, 2 mM EDTA, and 0.02% NaN_3. Gel filtration was carried out overnight at 4°C at a flow rate of 4 ml/h. Samples of 0.5 ml each were collected, analyzed for radioactivity and the radiolabeled DOL-C5b-7-containing fractions were pooled (2.5 ml).

DOL-C5b-8 vesicles: 0.5 ml DOL-^{125}I-C5b-7 vesicles (containing a total of 1 mg DOL and 50 μg C5b-7) were mixed with 15 μg ^{131}I-C8 (0.5 μCi) and incubated for 15 min at 37°C.

DOL-C5b-9 vesicles: 0.5 ml DOL-^{125}I-C5b-7 vesicles were mixed with 15 μg ^{131}I-C8 (0.5 μCi) and 15 μg C9 and incubated for 15 min at 37°C.

Sedimentation Coefficients of Intermediate Complexes. 0.2 ml DOL-C5b-7 vesicles containing ^{125}I-C5b-6 and ^{131}I-C7, 0.2 ml DOL-C5b-8 vesicles containing ^{125}I-C7 and ^{131}I-C8, and 0.2 ml DOL-C5b-9 vesicles containing ^{125}I-C7 and ^{131}I-C8 prepared as described above were dissolved with 0.2 ml 10% DOC, pH 8.1, 0.09 M NaCl, 0.02 M Tris-acetate, 2 mM EDTA, and 0.02% NaN_3. Incubation was allowed to proceed for 30 min at room temperature and the samples were then applied to 5 ml, 10–50% linear sucrose density gradients prepared in the same buffer containing 1% DOC and centrifuged as above. The gradients were fractionated into 15-drop fractions, the 11.5S C5b-6 complex, the 23S SC5b-9 complex, and the 33S MAC served as markers.

Electron Microscopy. 400-mesh copper grids coated with parlodion film (Pelco, Irvine, Calif.) and reinforced with carbon were used. The grids were made hydrophilic with bacitracin (11) (1 mg/ml) (Sigma Chemical Co.) before application of 5 μl sample. After excess sample was removed with the edge of a filter paper, the samples were stained with 1% sodium phosphotungstate (PTA), pH 7.2, or with 12 mM uranyl oxalate, pH 6.5. Grids were examined at 75 kV at 100,000-fold direct magnification in a Hitachi 12A microscope (Hitachi Ltd., Tokyo, Japan). The bright field image was recorded on a Kodak 4463 electron image film (Eastman Kodak Co., Rochester, N. Y.).

Results

Size and Shape of C5b-6. The C5b-6 complex has a 325,000 mol wt (4) as it is composed of one molecule of C5b and one molecule of C6. By electron microscopy, C5b-6 appears as an elongated, slightly curved structure (Fig. 1). Its approximate dimensions are 160 × 60 × 60 Å and the calculated vol of one molecule is 450,000 Å^3. This value agrees with the previously determined molecular weight of C5b-6 assuming a partial specific volume of 0.75 ml/g which corresponds to 1.4 Å^3/dalton.

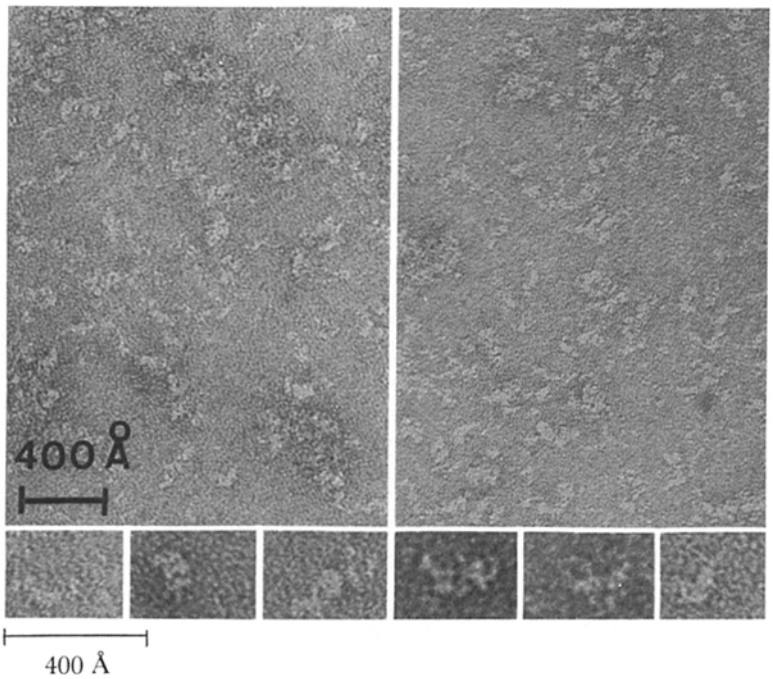

FIG. 1. Ultrastructure of isolated C5b-6. Protein concentration was 100 μg/ml and the sample was stained with uranyl oxalate.

concentration >1 mg/ml. C5b-6 paracrystals (Fig. 2) consist of parallel fibers, which may be arranged in helical strands.

Size and Shape of C5b-7. Upon mixing of C5b-6 with C7 in the absence of detergents or lipids, protein micelles are formed. C5b-7 micelles have an s of ~36S when examined by sucrose density-gradient ultracentrifugation which is indicative of a tetrameric composition (Fig. 3). This material, when visualized by electron microscopy (Fig. 4) reveals structures suggestive of a tetrameric nature. The C5b-7 monomer has the geometry of a half-ring whose outer and inner Diam are 200 and 100 Å, respectively. A short stalk of ~60 Å in length is attached to the center of the convex side of the half-ring. In the tetrameric micelles, C5b-7 monomers appear attached to each other through these 60-Å stalks.

C5b-7 bound to phospholipid vesicles (DOL) occurs in clusters and only occasionally are C5b-7 monomers seen (Fig. 5). Vesicle-bound C5b-7 also is visualized as a half-ring structure which is attached to the lipid bilayer apparently through the short stalk and parts of the half-ring are in contact with the surface. Upon extraction of C5b-7 from phospholipid vesicles with 10% DOC, C5b-7 has a sedimentation velocity of 36S when subjected to sucrose density-gradient ultracentrifugation in presence of 1% DOC suggesting that it is a tetramer (Fig. 6 a).

Size and Shape of C5b-8 and C5b-9. The tetrameric 36S C5b-7 complex is dissociated to an 18S complex by the binding of C8. Fig. 6 b shows an analysis of the C5b-8 complex by sucrose density-gradient ultracentrifugation after extraction with 10% DOC from DOL-vesicles. The major portion of the complex is seen to sediment as an 18S component. In addition, heavier material is present sedimenting with a velocity

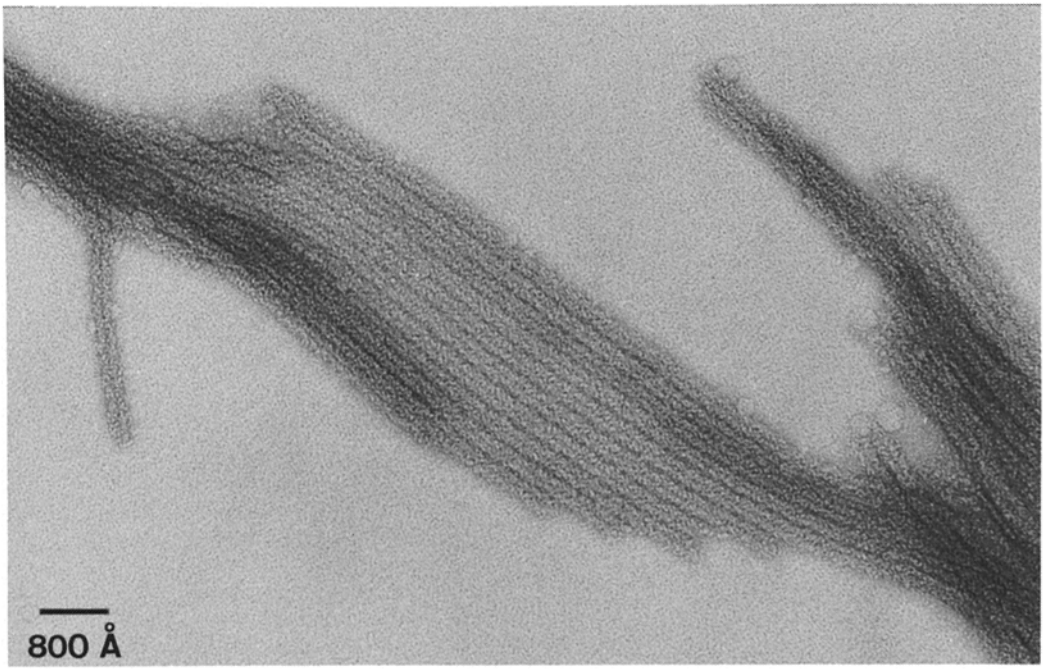

FIG. 2. Ultrastructure of paracrystals of C5b-6. Paracrystals were suspended at 1 mg/ml, cross-linked with 0.1% (final concentration) glutaraldehyde for 30 min at room temperature and then stained with uranyl oxalate.

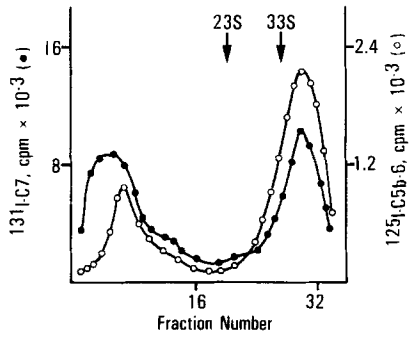

FIG. 3. Sucrose density gradient ultracentrifugation of C5b-7 protein micelles in the absence of detergents or lipids. Sedimentation is to the right and the position of markers is indicated by arrows. The 36S component was examined by electron microscopy.

of 25S and greater. The 18S component is interpreted to represent C5b-8 monomers, whereas the heavier material may constitute C5b-7 micelles with bound C8 that were incompletely dissociated. Binding of C9 to C5b-8 results in formation of dimers that have a sedimentation rate of 33.5S in accordance with previously reported results (1). Fig. 6c shows the ultracentrifugal analysis of C5b-9 extracted from DOL-vesicles with 10% DOC. In addition to 33S C5b-9, a minor amount of 23S C5b-9 is seen which is interpreted to constitute monomeric C5b-9.

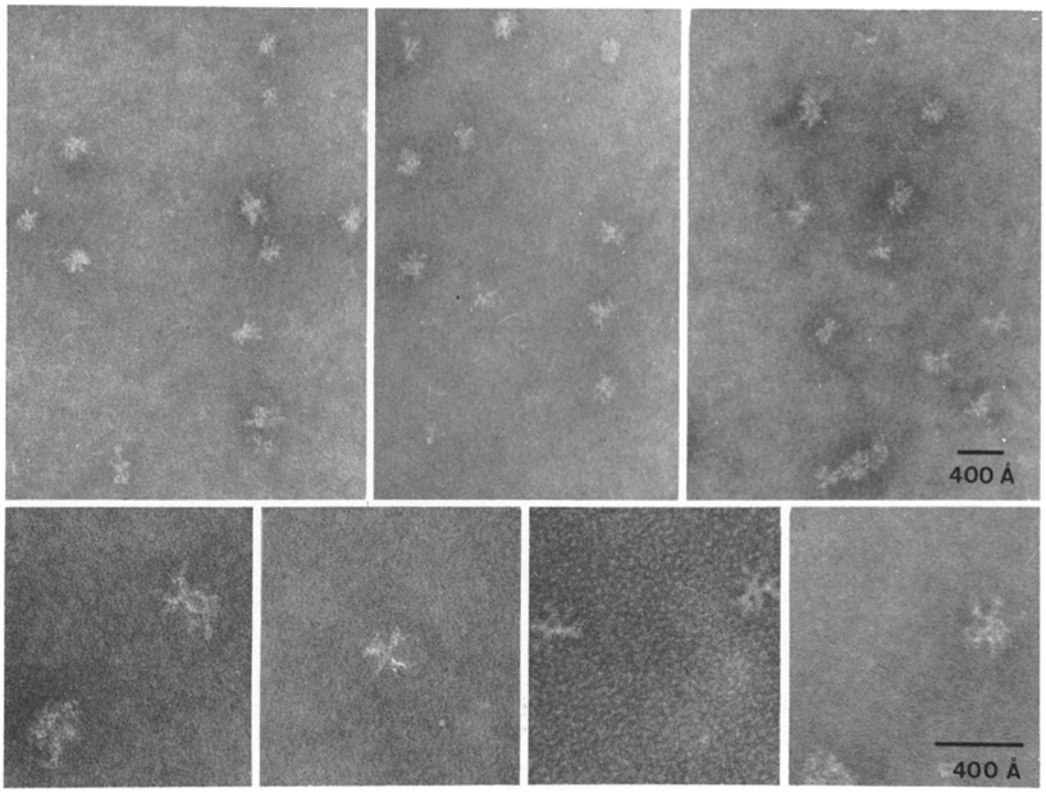

FIG. 4. Ultrastructure of C5b-7 protein micelles after sucrose density gradient ultracentrifugation and electrophoretic concentration. Protein concentration was 0.1 mg/ml and 1% PTA was used as negative stain.

Ultrastructurally, C5b-8 on DOL-vesicles appears as a half-ring with dimensions characteristic of C5b-7 which, however, is raised above the surface of the vesicle and attached to it through a 100-Å long stalk (Fig. 7). C5b-8 complexes also occur in clusters, which, unlike the C5b-7 clusters, are partially dissociable by 10% DOC (Fig. 6b). Upon addition of C9 (Fig. 8), typical complement lesions are formed on the DOL-vesicles which correspond to the ultrastructural image of the isolated dimeric MAC (1). Structures which apparently represent C5b-9 monomers can also be seen. Fig. 9a depicts a single C5b-9 monomer bound to a vesicle 800 Å in Diam. Fig. 9b shows a single dimer attached to a similar vesicle, whereas Fig. 9c depicts a vesicle 280 Å in Diam bearing a dimer and a monomer (top). Fig. 9d shows two apparently fused dimers on a 360-Å vesicle. The stalk in the C5b-9 monomer appears considerably wider than in the C5b-8 monomer, possibly because the half-ring of the C5b-9 monomer is attached through two stalks to the surface rather than one. When multiple MAC are bound to one DOL-vesicle, large structural defects in the continuity of the lipid bilayer become apparent (Fig. 9e, f). This process may approach the state where lipid bilayers are completely disassembled by the MAC. In addition, some MAC are visualized detached or in the process of becoming detached from the lipid bilayer (Fig. 8).

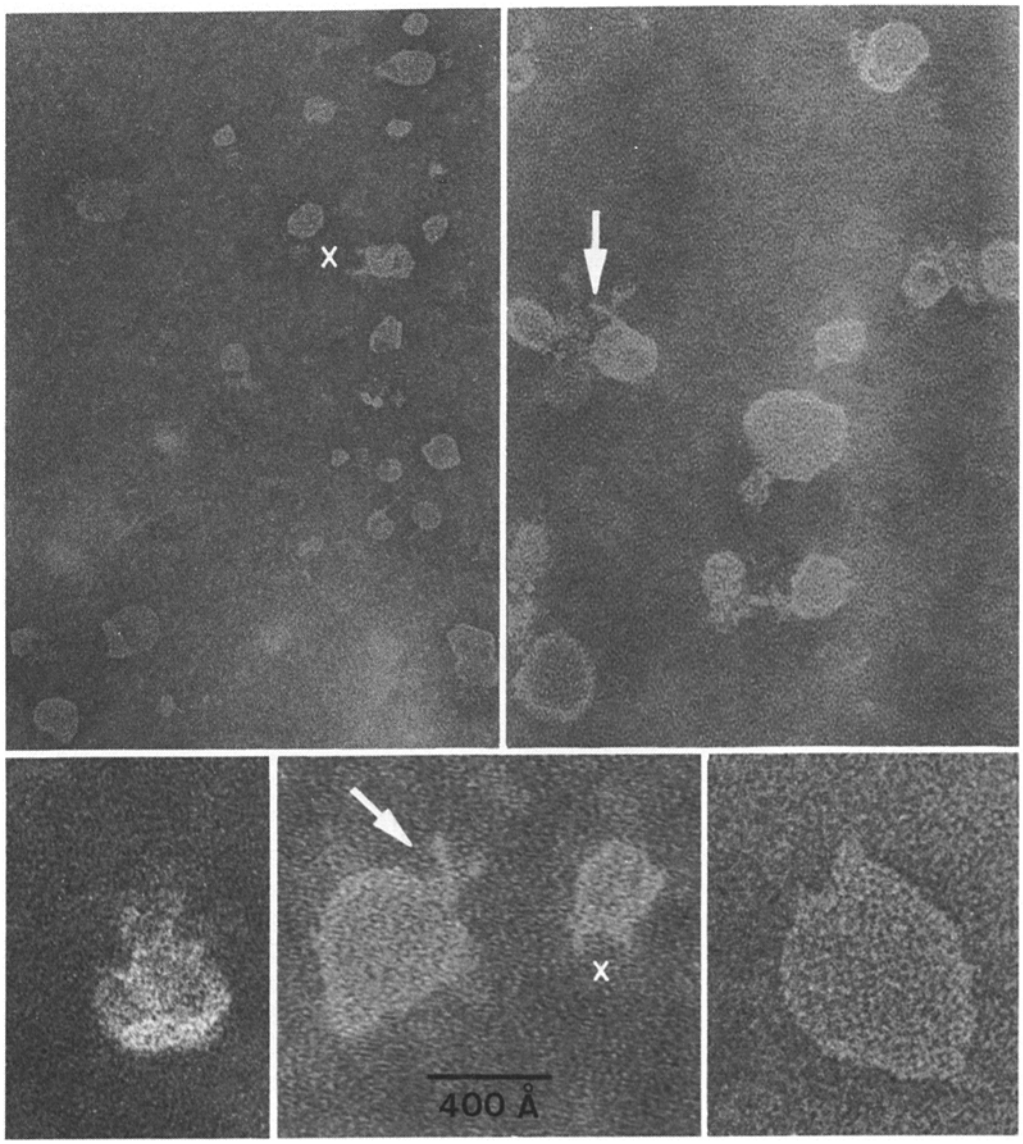

FIG. 5. Ultrastructure of C5b-7 bound to DOL-vesicles. DOL-vesicles bearing C5b-7 were prepared as described in Materials and Methods and stained with 1% PTA. The crosses indicate C5b-7 complexes at the level of the membrane, the arrows point to complexes attached to the bilayer apparently through the short stalk.

Discussion

These studies demonstrate that MAC assembly on lipid bilayer membranes is a complex reaction and that at each stage of assembly the complex exhibits a characteristic morphology and size. Binding of C7 to C5b-6 converts these two hydrophilic precursors to an amphiphilic complex, as was shown previously by detergent- and phospholipid-binding studies (12, 19). We now demonstrate that, in absence of detergents and lipids, the C5b-7 complex forms protein micelles, which are soluble in

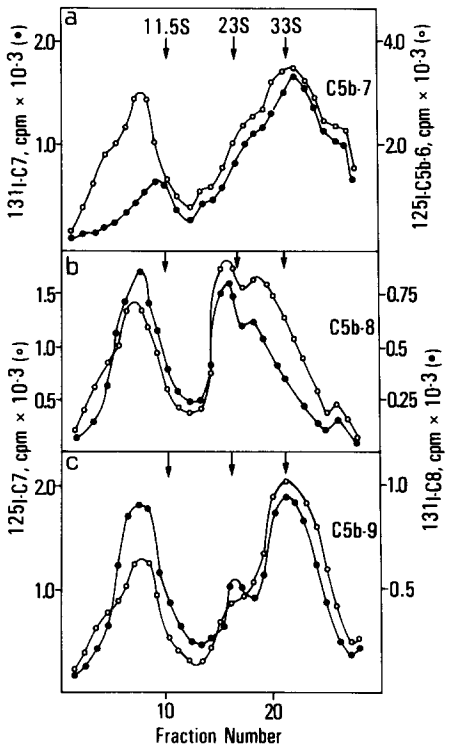

FIG. 6. Sucrose density-gradient ultracentrifugation of C5b-7, C5b-8, and C5b-9 extracted from DOL-vesicles with DOC. (a) C5b-7 containing ^{125}I-C5b-6 and ^{131}I-C7. (b) C5b-8 containing ^{125}I-C7 and ^{131}I-C8. (c) C5b-9 containing ^{125}I-C7 and ^{131}I-C8. The complexes were formed on DOL-vesicles and extracted with 10% DOC as described in Materials and Methods. The lower molecular weight material represents unreacted components. Sedimentation is to the right and the arrows indicate the position of reference proteins.

aqueous media, as has been described for amphiphilic membrane proteins (13). The electron microscopic examination and hydrodynamic measurements suggest a tetrameric structure of the C5b-7 protein micelles and allow an interpretation according to which four C5b-7 complexes each having the shape of a half-ring are aggregated via the short stalks extending from the convex side of the half-rings thus giving rise to a snowflake-like appearance. This morphology suggests that the hydrophobic domain of the amphiphilic C5b-7 complex is located in the short connecting stalk. Ultracentrifugal examination of C5b-7 extracted from DOL-vesicles suggests that C5b-7 bound to lipid bilayers also occurs as an oligomeric aggregate, probably a tetramer, which is not dissociated by 10% DOC. Although the phospholipid bilayer covers up the binding site, it is clear that not only the short stalk of C5b-7, but also the half-ring is in close contact with the membrane surface. Strong interaction of C5b-7 with the polar surface of lipid bilayers has previously been documented by electron paramagnetic resonance spectroscopy (14).

The binding of C8 to DOL-vesicle-bound C5b-7 has two effects: it raises the half-ring above the level of the membrane and it deaggregates the snowflake-like protein micelle. Whereas the sedimentation rate of the DOC-extracted C5b-7 complex is ~36S, that of the extracted C5b-8 complex is only 18S. Apparently in binding to the

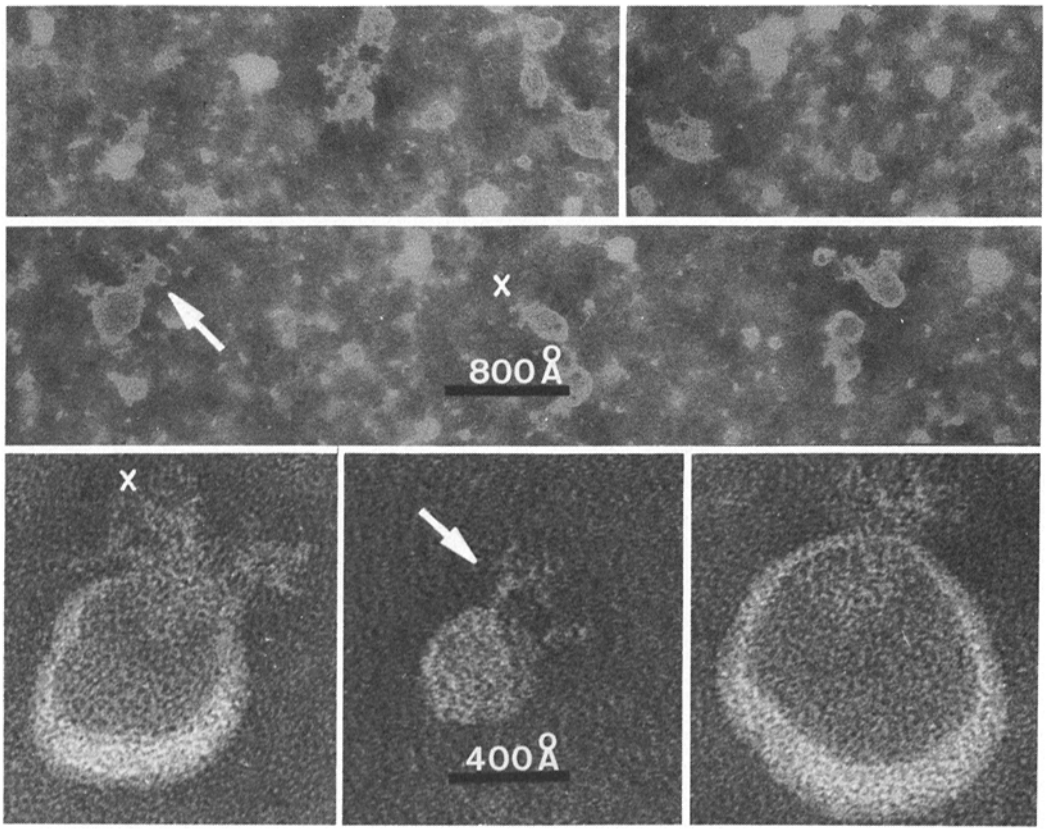

FIG. 7. Ultrastructure of C5b-8 bound to DOL-vesicles. Note the raised half-rings (arrows) and the formation of clusters (crosses). Sample was prepared as described in Materials and Methods and stained with 1% PTA.

C5b-7 protein micelle, C8 competes for the hydrophobic domains that are responsible for micelle formation. As a result, the micelle is dissociated and the individual C5b-8 complex appears now as a half-ring with a 100-Å-long and 30-Å-wide stalk. These observations suggest that C8 attaches to the short stalk of the C5b-7 complex and becomes part of the larger stalk of the C5b-8 assemblage. It is at this stage of MAC assembly that the complex penetrates into the hydrophobic interior of the lipid bilayer (14); nevertheless, the half-ring which is thought to be composed of C5b, C6, and C7 becomes removed from the surface of the membrane.

Binding of C9 has a pronounced effect on the ultrastructural image of the vesicle bound complex: the typical, ring-shaped complement lesions are now formed. This process is accompanied by an increase in the sedimentation rate of the DOC-extracted complex from 18 to 33S, indicating that C9 induces dimerization of two C5b-8 monomers. The failure of dimer formation upon binding of C9 to C5b-8 may be a result of a low density of C5b-9 monomers on the surface of vesicles or cells. C5b-9 monomers on phospholipid vesicles are seen in Fig. 9 and their occurrence may be ascribed to the attachment of a single complex or of an odd number of complexes to the vesicle.

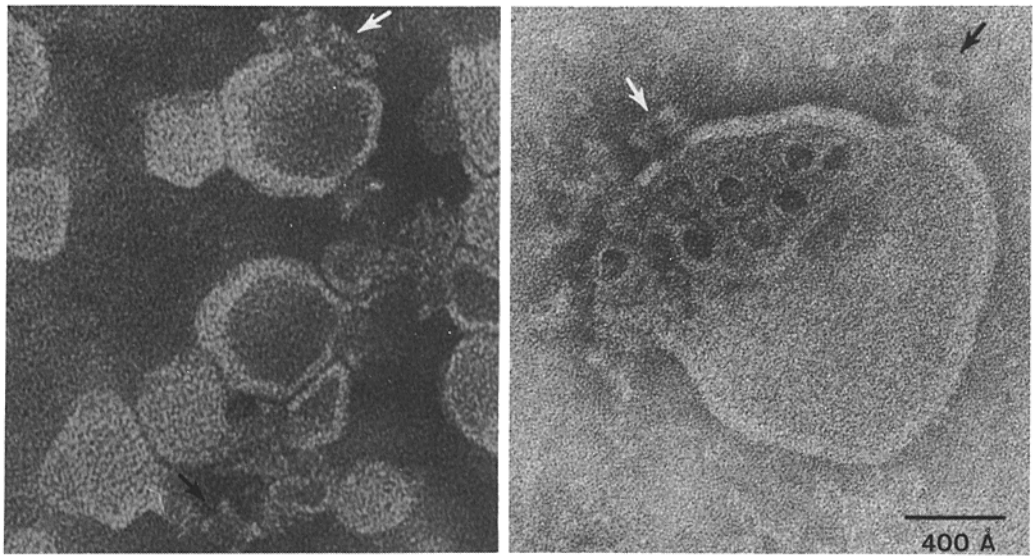

FIG. 8. Ultrastructure of C5b-9 bound to DOL-vesicles. Side views (white arrows) and top views (cross) of typical complement lesions are seen. Note also that some MAC are detached (black arrows).

On the basis of information obtained in this study and from earlier results (1, 15), the reaction of MAC formation may be formulated as follows:

$$\begin{aligned}
\text{C5} &\xrightarrow{\text{C5 convertase}} \text{C5a} + \text{C5b*} \\
\text{C5b*} + \text{C6} &\longrightarrow \text{C5b-6} \\
\text{C5b-6} + \text{C7} &\longrightarrow \text{C5b-7*} \\
4\text{C5b-7*} &\longrightarrow (\text{C5b-7})_4 \\
(\text{C5b-7})_4 + 4\text{C8} &\longrightarrow 4\text{C5b-8} \\
2\text{C5b-8} + 6\text{C9} &\longrightarrow 2\text{C5b-9} \\
2\text{C5b-9} &\longrightarrow (\text{C5b-9})_2,
\end{aligned}$$

where the asterisks denote metastable intermediate forms and where the micellar aggregate of the amphiphilic C5b-7 complex is assumed to be a tetramer. It may be speculated that the tendency of aggregation exhibited by C5b-7 may provide the topographical conditions necessary for eventual dimerization of C5b-9.

Dourmashkin (16) conducted an electron microscopic study of the events occurring at the erythrocyte membrane during reactive lysis by complement. Foliaceous particles of several hundred Å in Diam were seen at the C5b-7 stage that enlarged to particles with a variable number of arms at the C5b-8 stage. The use of erythrocytes in Dourmashkin's study favored the formation of clusters of the intermediate complexes, whereas DOL-vesicles of small diameter used by us allowed visualization of individual complexes including monomeric C5b-9.

Fig. 10 depicts a schematic representation of our results regarding the ultrastructural events occurring during MAC assembly. The slightly curved and elongated C5b-6 complex measuring 160 × 60 Å fuses with C7 to form a semicircular structure measuring 200 × 50 Å and having a 60-Å appendage at the center of the convex

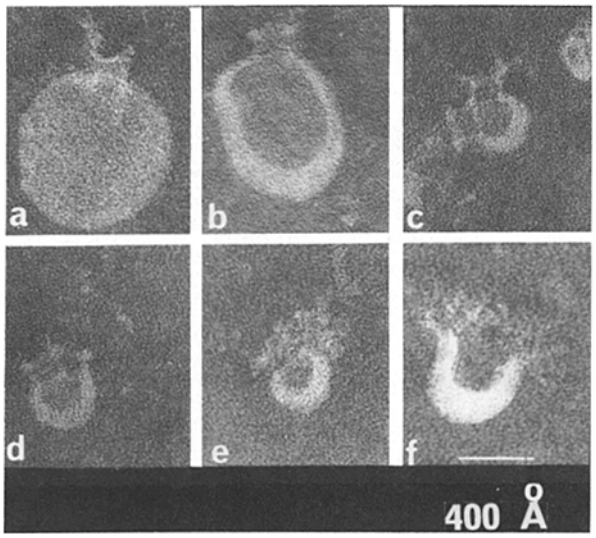

FIG. 9. Ultrastructure of several structural states of C5b-9 on single DOL-vesicles; (a) C5b-9-monomer; (b) C5b-9-dimer; (c) C5b-9 monomer and C5b-9 dimer; (d) two fused dimers in side view; (e) and (f) multiple C5b-9 per vesicle; note the structural defects of the lipid bilayer.

portion of the half-ring. Whereas this structure tends to aggregate to form soluble or membrane-bound tetramers, the attachment of C8 either prevents aggregation or causes deaggregation of protein micelles. The C5b-8 complex consists of a half-ring measuring 200 × 50 Å and having an appendage of 100 × 30 Å perpendicularly attached to it. C8 incorporation not only increases the length of the stalk, but raises the half-ring structure above the plane of the membrane. Upon binding of C9, the perpendicular stalk of the complex increases in size to 100 × 80 Å. Microenvironmental circumstances allowing, C5b-9 then dimerizes to the 1.6 × 10^6-dalton MAC which is composed of a closed 200- × 50-Å ring and two perpendicular attachments measuring 100 × 80 Å each. The proposed structure bears some relationship to the order of the previously described reversible interactions between the precursor proteins (17): C5 interacts with C6, C7, and C8, and C8 interacts with C9.

Although a description of the MAC assembly has not been put forth heretofore, a definite interpretation of MAC structure has been advanced. Tranum-Jensen et al. (3) as well as Dourmashkin (16) have described the MAC as a hollow cylinder projecting from the cell membrane and partly penetrating it. The hollow cylinder model has been linked to Mayer's (18) doughnut hypothesis of complement-dependent cell lysis, according to which C5b-9 produces a hydrophilic transmembrane protein channel.

Our work does not refute the protein channel hypothesis, rather it seems likely that the MAC does contain some form of an internal channel. However, in regard to the mechanism of action of the MAC, it is important to emphasize a clearly established function of the MAC. As the MAC assembles, its intermediate complexes acquire increasing numbers of phospholipid binding sites, the fully assembled MAC having the capacity of binding ~1,500 phospholipid molecules (19). This means that the formation of MAC protein-phospholipid complexes in membranes greatly reorganizes the orientation of lipid molecules (14). At the contact region between ordered bilayer

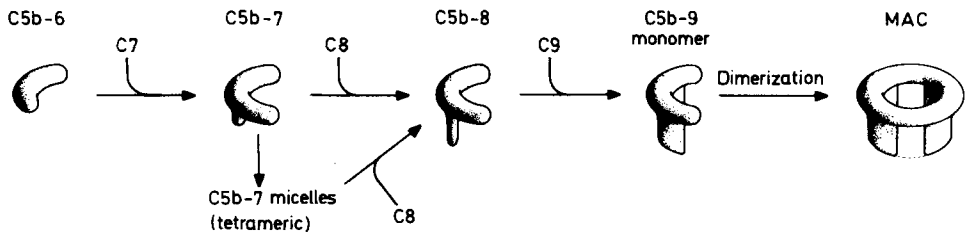

FIG. 10. Schematic representation of structural aspects of MAC formation.

structure and the more micellar lipid-protein complexes, the normal permeability barrier of membranes may break down and leaks may occur. These membrane leaks occur independently of the circular or cylindrical C5b-9 dimer. Dependent upon membrane fluidity, they may be caused by C5b-7 (20) or C5b-8 (21, 22) as well as by the MAC. In fact, liposomal membranes, viral membranes (23) and bacterial cell walls (24) may be disassembled by the MAC independently of formation of transmembrane channels.

This work constitutes part of our endeavor to elucidate the architecture of the MAC and the process of protein fusion that results in MAC formation and in the generation of membranolytic activity. Work is underway to delineate the three-dimensional structure of the MAC and to assign within the MAC structure distinct topological positions to the immunochemically detectable precursor proteins.

Summary

This study was conducted to gain insight into the process of assembly of the membrane attack complex (MAC) of complement through structural analysis. Four intermediate complexes and the MAC were examined by electron microscopy and by sucrose density-gradient ultracentrifugation. The C5b-6 complex has a sedimentation rate of 11S, an elongated, slightly curved shape and dimensions of 160 × 60 × 60 Å. At protein concentrations >1 mg/ml, and physiologic ionic strength and pH, the complex forms paracrystals that have the appearance of parallel strands. Equimolar quantities of C5b-6 and C7 mixed in the absence of lipids or detergents give rise to C5b-7 protein micelles which are soluble in aqueous media and have a sedimentation rate of 36S, suggesting a tetrameric composition. Ultrastructurally, C5b-7 protein micelles consist of four half-rings, each measuring 200 × 50 Å, which are connected to one another by short stalks extending from the convex side of the half-rings. C5b-7 bound to dioleoyl lecithin (DOL) vesicles has a similar ulrastructural appearance. After extraction with deoxycholate (DOC), C5b-7 has a sedimentation velocity of 36S which further suggests the occurrence of C5b-7 in the form of tetrameric protein micelles. Attachment of C8 to vesicle-bound C5b-7 results in dissociation of the protein micelles. An individual C5b-8 complex appears as a half-ring attached to the DOL-vesicle via a 100-Å-long and 30-Å-wide stalk. After extraction from the DOL-vesicles with DOC, C5b-8 has a sedimentation velocity of ~18S. Binding of C9 to DOL-vesicle bound C5b-8 induces the formation of the typical ultrastructural com-

plement lesions. C5b-9 extracted from the vesicles with DOC has a sedimentation rate of 33S, which is characteristic of the C5b-9 dimer. It is concluded that dimerization is a function of C9. C5b-9 monomers are visualized when a single C5b-9 complex or an odd number of complexes were bound per DOL-vesicle. The C5b-9 monomer has an ultrastructural appearance that is theoretically expected of a half-dimer: a 200- × 50-Å half-ring which is attached to the DOL-vesicle by a 100- × 80-Å appendage. Extracted with DOC, the C5b-9 monomer has a sedimentation rate of 23S. At a higher multiplicity of MAC per DOL-vesicle, large structural defects in the lipid bilayer are seen which are attributed to direct physical destruction of membranes by the known lipid-binding capacity of the MAC. It is proposed that protein micelle formation at the C5b-7 stage of MAC assembly and dissociation of these micelles upon binding of C8 are events that facilitate dimerization of C5b-9 and thus MAC formation.

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