Proc. Natl. Acad. Sci. USA
Vol. 75, No. 11, pp. 5655–5659, November 1978
Immunology

Molecular nature of the complement lesion

(complement lysis/C5b-9 complex/membrane reconstitution/transmembrane pore/electron microscopy)

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Communicated by K. Frank Austen, July 25, 1978

ABSTRACT **The principle molecular event leading to membrane perturbation by complement is the assembly of the terminal five serum complement components (C5b–C9) into a macromolecular C5b-9 complex on the target membrane [Müller-Eberhard, H.-J. (1975) *Ann. Rev. Biochem.* 44, 697–723]. The present communication reports on the ability of purified C5b-9 complexes isolated from target membranes to become reincorporated into artificial lipid vesicles. The data indicate that the complex is a vertically oriented, hollow, cylindrical macromolecule possessing lipid-binding regions that enable one terminus to penetrate into the lipid bilayer. A transmembrane pore appears to be created at the attachment site of the C5b-9 complex.**

Assembly of the terminal five complement components (C5b–C9) into a C5b-9 complex on a target membrane forms the molecular basis of the membrane attack mechanism of complement (1–5). We have shown that this membrane C5b-9 complex [C5b-9(m)] derived from human serum is a cylindrical macromolecule, identical to the classical ultrastructural membrane "lesion" of complement (6–8). Two lines of evidence have emerged which indicate that C5b-9(m) penetrates into the lipid matrix. First, it resembles "integral" membrane proteins (9, 10) in that (*i*) it requires detergents for elution from membranes (11–13) and (*ii*) it is amphiphilic and binds Triton X-100 (14–16). Second, ultrastructural studies directly implicate partial burial of an annular terminus within the membrane bilayer (6). A membrane discontinuity is observable at the attachment site of the complex after proteolytic removal of membrane-associated proteins. In agreement with Mayer's "doughnut" hypothesis (17) we have therefore proposed that the C5b-9(m) complex damages the membrane by penetrating into the lipid bilayer to form a transmembrane pore (6).

Under appropriate conditions, "integral" membrane proteins may interact with lipid through their apolar, detergent-binding domains and become reincorporated into artificial lipid vesicles (18–22). This study describes such membrane reconstitution experiments conducted with isolated C5b-9(m) complexes. The results show that the complex binds lipids and indicate that attachment of C5b-9(m) indeed results in the formation of a transmembrane pore.

MATERIALS AND METHODS

Sources of reagents and antisera were as described (6, 11, 14, 23). Native and proteolyzed C5b-9(m) were isolated as described (6, 23). The phosphate buffer used for chromatography (6) was replaced by 10 mM Tris/50 mM NaCl/15 mM NaN_3 buffer, pH 8.2; detergent was added as before (6). Native C5b-9(m) was additionally prepared by a simplified procedure through chromatography of Triton extracts of membrane over Sepharose 6B in 0.05% Triton X-100 and rechromatography of the pooled and concentrated C5b-9(m)-containing fractions over Sepharose 6B in Triton X-100 plus deoxycholate. Fractions containing the monomerized complexes were contaminated with three or four serum proteins, but this did not affect the reconstitution experiments.

Reincorporation of C5b-9(m) into Lipid Vesicles. Lipid vesicles were prepared through a deoxycholate dialysis procedure (18). The most satisfactory preparations of reconstituted vesicles were obtained by using a mixture of deoxycholate-solubilized sheep erythrocyte membrane lipids. Packed, washed sheep erythrocyte membranes prepared by hypotonic lysis in 5 mM phosphate (pH 8.0) (24) (protein concentration, 3–4 mg/ml) were solubilized in 1% (wt/vol) deoxycholate. Aliquots (3.5 ml) of solubilized membrane samples were layered onto 10–50% linear sucrose density gradients (5 mM Tris/25 mM NaCl/8 mM NaN_3, pH 8.2, containing 0.1% deoxycholate; gradient volumes, 8.5 ml) and centrifuged at 37,000 rpm for 30 hr at 4°C in a Spinco ultracentrifuge L2 65B (rotor type SW 41 Ti). Thereafter, the deoxycholate-solubilized membrane lipids were recovered in the top 3.4 ml of the gradients. The preparations contained less than 70 μg of protein per ml, as determined by amino acid analyses. The lipid content of the samples was approximately 1 mg/ml, as determined by dry weight measurement after dialysis against distilled water for 2 days. The presence of the major classes of membrane lipids was confirmed by thin-layer chromatography.

One volume of freshly prepared native or proteolyzed C5b-9(m) (2–3 ml) was added to 1 vol of deoxycholate-solubilized lipids and the samples were dialyzed against 300 vol of 4 mM Tris/5 mM NaN_3, pH 8.0, at 25°C for 2 hr. $MgCl_2$ was then added to a final concentration of 3 mM, and dialysis was continued for another 24 hr. The C5b-9(m) preparations were taken directly from the Sepharose columns used for their isolation (6). Protein concentrations as determined by amino acid analyses were generally in the order of 200 μg/ml. In some experiments, lower protein concentrations were used, resulting in a lower density of complex incorporation into the lipid vesicles.

Dialyzed samples were concentrated to approximately $1/10$th volume by packing the dialysis bags into Sephadex G-100 for several hours. Aliquots were examined in the electron microscope. The samples were made 45% in sucrose, overlayered with 3 ml of 40% sucrose and 1 ml of 10% sucrose in the same reconstitution buffer, and centrifuged for 15 hr at 200,000 × *g* (rotor type, SW 65Ti). Collected samples were examined by rocket immunoelectrophoresis. Dialyzed aliquots were examined in the electron microscope.

Other Methods. Quantitative immunoelectrophoresis and sodium dodecyl sulfate gel electrophoresis were performed as in refs. 11 and 23, respectively. Lipid extractions with chloroform/methanol and thin-layer chromatography were performed as in ref. 25.

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Abbreviations: C5b-9, complex of terminal five complement components; C5b-9(m), membrane C5b-9 complex.

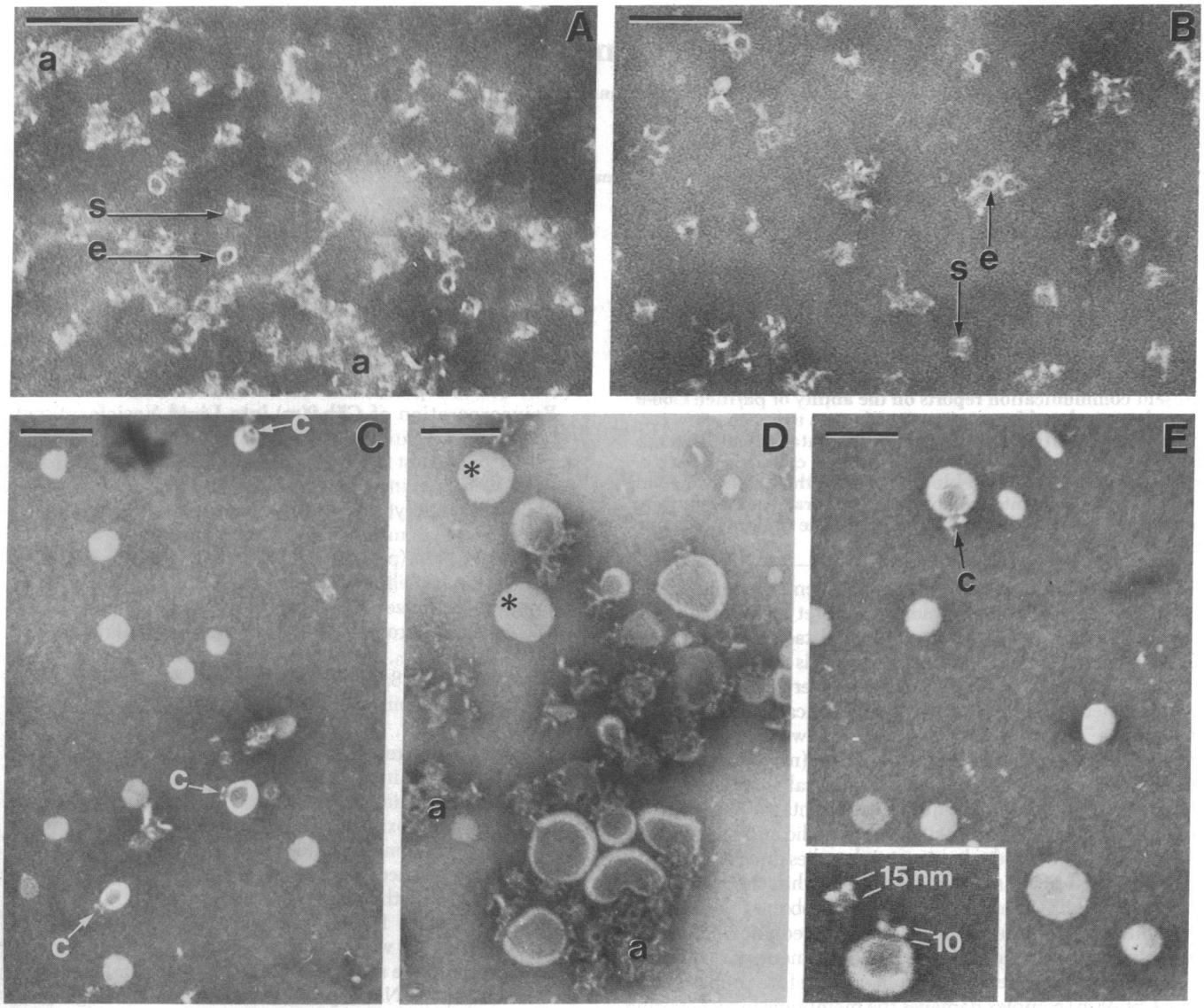

FIG. 1. Electron micrographs of samples negatively stained with sodium silicotungstate. Scale bars indicate 100 nm in all figures. (*A*) Isolated C5b-9(m) not proteolyzed. The cylindrical macromolecules are seen in side-projections (s) and along the cylindrical axis (e). Some aggregates are seen (a). (*B*) Isolated, proteolyzed C5b-9(m), showing the unaltered structure of the complex. (*C*) Lower power survey of a preparation of lipid vesicles reconstituted with proteolyzed C5b-9(m). Reincorporated complexes (c) can be seen in side and axial projections. The interiors of lipid vesicles not carrying complexes typically show uniform low density. (*D*) Concentrated preparation of lipid vesicles formed in the presence of a higher concentration of C5b-9(m). Many of the vesicles carry several complexes. Vesicles not carrying complexes (asterisks) are empty of stain. a, aggregates of C5b-9(m). (*E*) Lipid vesicles reconstituted with proteolyzed C5b-9(m). The micrograph depicting many complex-free vesicles was selected to demonstrate the consistent difference in stain-filling between vesicles with and without the reincorporated complex. (*Inset, Lower Left*) Note 5-nm height difference of the C5b-9(m) cylinder measured in isolation and on the vesicle membrane.

Electron Microscopy. Negative staining with 2% sodium silicotungstate at pH 7.5 or 2% sodium phosphotungstate at pH 6.8 and electron microscopy were performed as described (6). The osmolality of the staining solutions was measured by freezing-point depression and chosen to be slightly hyperosmotic to the suspending medium of the lipid vesicles.

RESULTS

Fig. 1 *A* and *B* are electron micrographs of the native and proteolyzed C5b-9(m) preparations used for the illustrated reconstituted experiments. No membrane lipids could be detected by thin-layer chromatography of chloroform/methanol extracts prepared from these preparations. This indicates that chromatography of the complex in the presence of detergent results in extensive delipidation, as has been described for other amphiphilic membrane proteins and serum lipoproteins (15, 26). As reported (6), the complex was a short (15 nm), thin-walled cylinder (apparent internal diameter, 10 nm) rimmed by a broader annulus at one end. No structural differences have been observed between native and proteolyzed complexes, but native C5b-9(m) displayed a slightly higher tendency to aggregate. These solubilized complexes were added to lipid/detergent solutions. During subsequent removal of deoxycholate through dialysis, a fraction of the complexes entered into molecular aggregates, most of which displayed no recognizable order (Fig. 1*D*). A number of complexes interacted with lipid to become incorporated into the vesicles that formed. Identical results were obtained with both native and proteolyzed complexes (Fig. 1 *C* and *E*). In various experiments, and dependent on the concentration of complexes present during reconstitution, 50–80% of the vesicle carried the incorporated complexes. Some vesicles carried low numbers (one to three) (Fig. 1*C*), whereas others were laden with high numbers of complexes (Fig. 1*D*).

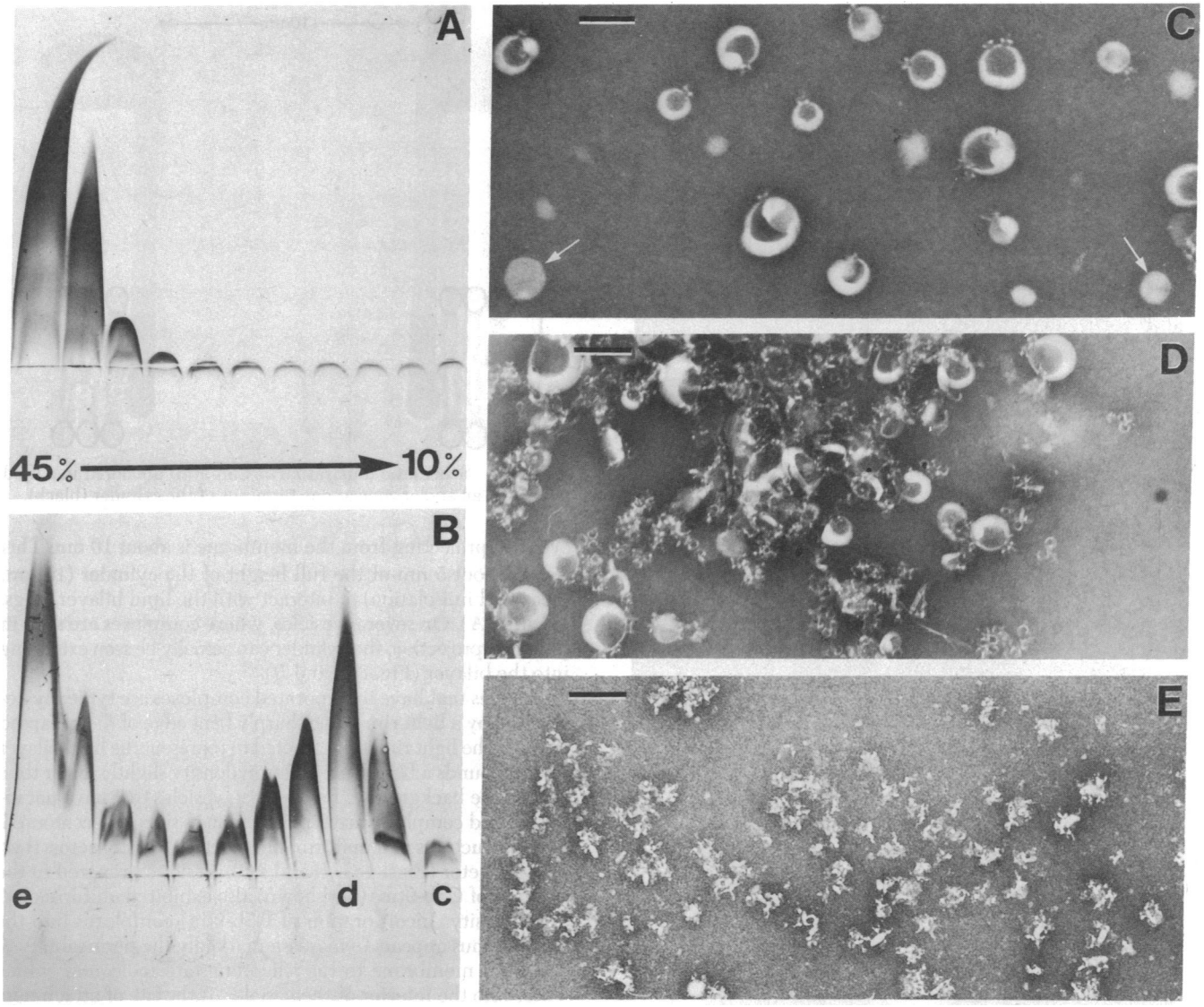

FIG. 2. (*A* and *B*) Rocket immunoelectrophoresis of fractions obtained after flotation of lipid vesicles and C5b-9(m) through sucrose gradients. (*A*) When dialyzed in the absence of lipid, C5b-9(m) remained at the bottom of the 45–10% sucrose gradient during centrifugation. (*B*) When dialysis was performed together with lipid, a fraction of the complexes floated in association with the lipid vesicles that formed. Samples applied were 20 μl in *A* and 30 μl in *B*. The total amount of C5b-9(m) distributed across the gradient in *B* was 500 μg. Rocket immunoelectrophoresis was performed by using 10 μl of antiserum to C5b-9(m) "neoantigens" per cm^2 (14). Agarose gels contained 0.5% Triton X-100. (*C*) Electron micrograph of the top fraction (c) of the sucrose gradient of *B*. This fraction contained lipid vesicles that had incorporated single C5b-9(m) complexes and were filled with stain, together with vesicles that had escaped complex incorporation and appeared to be empty (arrows). The stain continuity through the internal diameter of the C5b-9(m) cylinders and into the lipid vesicles is clearly seen in several cases. (*D*) Electron micrograph of fraction d of *B*, depicting aggregates of distorted lipid vesicles carrying massive numbers of complexes. (*E*) Only aggregates of the complex were recovered from the bottom fractions (e) of the gradient.

Flotation of the reconstituted vesicles through sucrose density gradients confirmed the association of C5b-9(m) with lipids. Results obtained with both native and proteolyzed C5b-9(m) were identical, and a representative example is shown in Fig. 2. When the complex was dialyzed in the absence of lipids, concentrated, and centrifuged, all the material remained at the bottom of the gradient during centrifugation (Fig. 2*A*). When dialysis was performed in the presence of lipid, subsequent centrifugation resulted in flotation of a fraction of C5b-9(m) with the vesicles through the gradient. In different experiments, approximately 50–90% of the complexes became lipid-associated, as estimated by planimetric quantitation of the areas delimited by the immunoprecipitates (Fig. 2*B*). Fig. 2 *C–E* show electron micrographs of fractions obtained from such a sucrose gradient. The bottom fractions contained solely aggregated C5b-9(m) (Fig. 2*E*). The fractions in the upper part of the gradient contained vesicles to which large numbers of C5b-9(m) were attached (Fig. 2*D*). These vesicles aggregated somewhat during concentration and centrifugation. The uppermost fractions of the gradient contained vesicles reconstituted with small numbers of complexes, as well as complex-free vesicles (Fig. 2*C*).

Sodium dodecyl sulfate gel electrophoresis of vesicle fractions containing C5b-9(m) revealed protein bands that corresponded to the gel pattern of isolated C5b-9(m) (not shown). Immunochemical analyses also showed that C5 and C9 were complexed to one another on the reconstituted vesicles (not shown). The unaltered reaction of reincorporated C5b-9(m) with the antiserum to complex "neoantigens" (14) can be discerned from the rocket immunoelectrophoresis (Fig. 2). Thus, there is no evidence to suggest a dissociation and selective incorporation of only a part of the complex into the lipid vesicles during the reconstitution experiments.

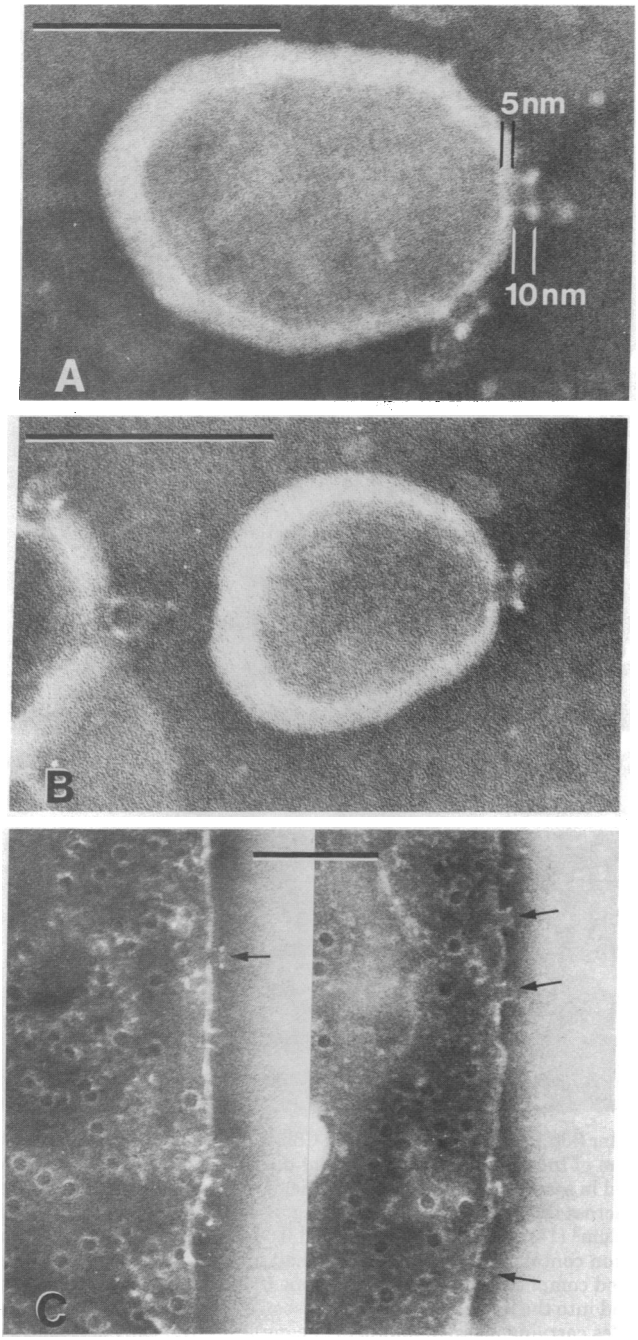

FIG. 3. (*A*) Reincorporated complex (top) in exact side projection. It extends through the full thickness of the light rim, interpreted to represent the bent edge of the lipid membrane, which is attenuated at the complex attachment site. (*B*) Light rim is clearly interrupted at this site. (*C*) Proteolyzed membranes of complement-lysed sheep erythrocytes, showing the identical orientation of the complex on the target membrane (see ref. 6). C5b-9(m) complexes are seen in axial projections over the ghost membrane and in side projections (arrows) along the bent membrane edge. Membrane discontinuities at complex attachment sites are often clearly seen. Scale bars indicate 100 nm.

Additional electron micrographs of lipid vesicles reconstituted with C5b-9(m) are shown in Fig. 3 *A* and *B*. The cylindrical axis of a given complex is oriented perpendicularly to the membrane, and the annulus has always been observed to be located external to the vesicle. The orientation of the complex reincorporated into lipid vesicles is thus morphologically identical to its orientation on target sheep erythrocyte membranes (compare with Fig. 3*C* and ref. 6). The height of the

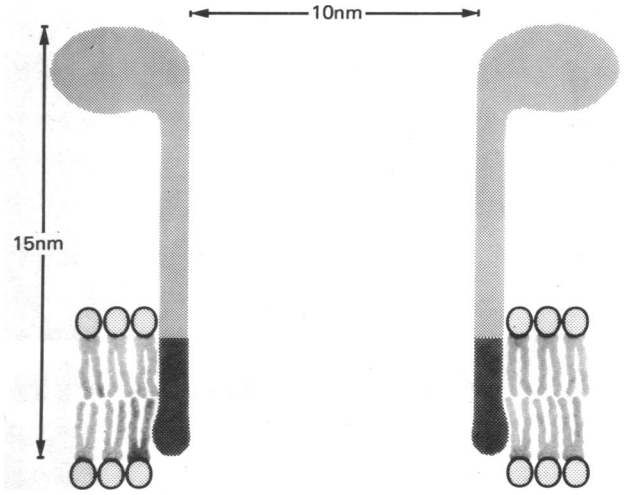

FIG. 4. Schematic illustration of C5b-9(m) anchored in a lipid bilayer by an apolar zone at one terminus of the cylinder (black).

cylinder projecting from the membrane is about 10 nm. This leaves about 5 nm of the full height of the cylinder (15 nm, measured in isolation) to interact with the lipid bilayer (Figs. 1*E* and 3*A*). On several vesicles, where complexes are seen in pure side projection, the cylinder can actually be seen extending into the bilayer (Fig. 3 *A* and *B*).

Vesicles that have incorporated complexes are typically delineated by a light rim at the sharply bent edge of the collapsed vesicle. The light rim is interpreted to represent the lipid bilayer and surrounds a field of an electron density slightly lower than that of the background. By contrast, vesicles that have not incorporated complexes are typically seen as smoothly contoured, round structures of a uniform, low electron density across their full diameter (Figs. 1 *C–E* and 2*C*). Vesicles prepared in the absence of C5b-9(m) (not shown) also exhibited uniform electron density. Incorporation of C5b-9(m) complexes into the vesicles thus appears to increase markedly the permeability of the lipid membrane to the silicotungstate molecules, which enter into the interior of the vesicle. At the site of attachment of the complex, the membrane is attenuated or frankly interrupted, so that continuity of stain deposits on either side of the light rim can be seen through the inner diameter of the stain-filled complexes (Figs. 3 *A* and *B* and 2*C*). Within the limits of ultrastructural image interpretation, these findings indicate the presence of true pores that are walled by the inserted C5b-9(m) complexes across the lipid bilayer.

DISCUSSION

The present study leads us to conclude that the C5b-9(m) complex of human complement possesses lipid-binding surfaces that enable it to penetrate into and possibly through the lipid bilayer of a target membrane (Fig. 4). This association probably occurs via the same molecular regions that bind detergent (14–16). The identical results obtained with native and proteolyzed C5b-9(m) indicate that both molecules possess essentially the same binding sites for lipid. These are apparently primarily confined to one terminus of the cylinder, which penetrates into the lipid bilayer. The annular rim, identical with the classical complement "lesion" (6), was never seen associated with the lipid bilayer. The height measurements of the C5b-9(m) cylinders in isolation and on the lipid vesicles provide further ultrastructural evidence that penetration of the complex into the lipid bilayer truly occurs. An interesting finding is that, in 15 experiments, the C5b-9(m) cylinder was never observed to project into the lumen of a lipid vesicle.

Isolated as well as membrane-bound complexes are consistently filled with stain. From this we deduce that the interior of the cylinder is hollow. The continuity of stain deposits outside a given lipid vesicle into and through a C5b-9(m) cylinder is interpreted to depict a true transmembrane channel walled by the complex, whereby the functional diameter of the pore is not defined. The observation that lipid vesicles carrying the complexes are always permeable to the silicotungstate stain (molecular weight, approximately 3000), which under identical conditions does not appreciably enter vesicles not carrying the complex, is in accordance with this molecular model.

In basic agreement with the "doughnut" hypothesis originally put forward by Mayer (17), we tentatively propose that formation of such a transmembrane pore walled by the C5b-9(m) complex constitutes the basic molecular mechanism of membrane perturbation by complement. This model explains the sole necessity of C5–C9 components for generation of the functional membrane lesion (1–3, 27). It accounts for the necessity of the presence of all C5–C9 components in the formation of the classical ultrastructural membrane "lesion" (28–30). It explains why complement lysis apparently does not involve degradation of membrane lipids, and why C5b-9 membrane attachment does not require the presence of "receptors" (27, 31, 32). It accounts for the nonelutability of the complex from target membranes with agents other than detergents (11–13). The concept of pore formation by C5b-9(m) also receives direct support from experiments involving measurements of electrical conductance across planar lipid bilayers during the action of complement (33). Finally, other C5b-9(m)-induced membrane phenomena, including nonosmotic membrane-swelling (34), membrane intercalar particle aggregation (35), and lipid release (36–39), are probably causally linked to the insertion of the terminal complement complex into the lipid bilayer.

Dr. H.-J. Wellensiek provided the excellent laboratory facilities in Giessen which rendered completion of this work possible. The technical assistance of Dorothea Sziegoleit, Barbara Hönig, and Kjeld Stubb during parts of these studies is gratefully acknowledged. We thank Dr. Ari Helenius (European Molecular Biology Laboratory, Heidelberg, West Germany) for valuable discussions and advice, Dr. Ole Bjerrum (The Protein Laboratory, Copenhagen, Denmark) for his enthusiastic interest in this work, and Dr. Manfred M. Mayer (The Johns Hopkins University, Baltimore, MD) for helpful suggestions and critical reading of this manuscript. The amino acid analyses were kindly performed by R. Warth at the Max Planck Institute for Immunobiology (Freiburg, West Germany). This study was supported by the Danish Medical Research Council through Grant 512-8465 to S.B. and by a subsequent grant from the Deutsche Forschungsgemeinschaft.

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