Terminal Membrane C5b-9 Complex of Human Complement: Transition from an Amphiphilic to a Hydrophilic State through Binding of the S Protein from Serum

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ABSTRACT   The membrane-damaging C5b-9(m) complex of complement is a cylindrically structured, amphiphilic molecule that is generated on a target membrane during complement attack. Isolated C5b-9(m) complexes are shown here to possess the capacity of binding a protein, termed "S"-protein, that is present in human plasma. Binding of this protein apparently shields the apolar surfaces of C5b-9(m), since the resulting "SC5b-9(m)" complex is hydrophilic and no longer aggregates in detergentfree solution. Dispersed SC5b-9(m) complexes exhibit an apparent sedimentation coefficient of 29S in sucrose density gradients, corresponding to a molecular weight of ~1.4 million. SDS PAGE analyses indicate binding of 3-4 molecules S-protein per C5b-9(m) complex. These data are consistent with a monomer nature and molecular weight of 1-1.1 million of the C5b-9(m) complex. Ultrastructural analysis of SC5b-9(m) shows preservation of the hollow cylindrical C5b-9(m) structure. Additional material, probably representing the S-protein itself, can be visualized attached to the originally membrane-embedded portion of the macromolecule. The topography of apolar surfaces on a molecule thus appears directly probed and visualized through the binding of a serum protein.

Complement-mediated membrane damage is caused by the attachment of the terminal C5b-9 complement components to the target lipid bilayer (17, 19, 26). These proteins assemble into a macromolecular C5b-9(m) protein complex which possesses the structure of a hollow cylinder that lies partially embedded within the membrane, probably creating a transmembrane channel (4, 18, 20, 27). The complex has been shown to be an amphiphilic molecule with properties typical of an integral membrane protein (12, 25): it is not eluted from the membranes by ionic manipulations (1, 13); it is solubilized by Triton X-100 (2, 29); it binds large amounts of detergent (3, 10), and also binds lipid (4, 7) to become reincorporated into artificial lipid vesicles. The formation of C5b-9(m) is unique in that it represents the only known case of such a transition of serum proteins from a hydrophilic to an amphiphilic state.

Complement activation can also occur in the absence of a target lipid bilayer, e.g., via the alternative pathway when particulate inulin is added to serum (14, 15). In these cases, a macromolecular complex consisting of C5b-C9(m) components forms in solution. However, another serum protein, termed the "S-protein" becomes additionally incorporated (16). The ensuing macromolecule, accordingly designated "SC5b-9", is a water-soluble, noncytolytic protein of 1,000,000 mol wt with no currently known biological function. Evidence indicates that the S-protein binds to the nascent terminal complex at the stage of C5b-7 assembly, whereby it is believed to bind to and mask apolar surfaces, thus abrogating the capacity of the trimolecular complex to hydrophobically interact with lipid bilayers (22). Compatible with this view is the finding that removal of the S-protein from SC5b-9 through high concentrations of deoxycholate (23) or by tryptic attack (5, 23) leads to exposure of lipid-binding surfaces on the macromolecule.

In the present study, we show that the S-protein can bind to preformed, C5b-9(m) complexes and hereby convert the primarily amphiphilic macromolecule to a hydrophilic state. Ultrastructural analyses locate the binding sites for the S-protein to the outer surface of the C5b-9(m) complex, and are consistent with previous conclusions regarding the topography of the apolar region on the protein cylinder (4, 6, 27). Hydrodynamic analyses indicate a molecular weight of 1.4 million for the SC5b-9(m) complex, supporting the contention that the C5b-9(m) complex is a monomer entity (19), rather than a dimer complex (11) of the C5b-9(m) components.

MATERIALS AND METHODS

Preparation of C5b-9(m)

Rabbit erythrocytes drawn in 10 mM EDTA (final concentration) were washed three times in saline. One volume of a 10% cell suspension in veronal-buffered

saline containing 1.0 mM Mg^{2+} and 0.15 mM Ca^{2+} was lysed at 37°C by the addition of 1 vol whole human serum (WHS). Lysis occurring through alternative complement activation (21) was complete within 3 min. Membranes were pelleted (Sorvall GSA rotor, 10,000 rpm, 15 min at 4°C: Du Pont Instruments, Newtown, CT) and washed three times in 5 mM sodium phosphate buffer, pH 8.0. The washed membranes were solubilized by the addition of solid sodium deoxycholate (Merck & Co., Inc., Rahway, NY, analytical grade) to a final concentration of 10% (wt/vol) (250 mM) and agitation at room temperature for 15–30 min (11). Aliquots of 3 ml of solubilized membrane material were applied to 36-ml, linear 10–50% sucrose density gradients prepared in 5 mM Tris-HCL, 25 mM NaCl, pH 8.2, containing 4 mM deoxycholate. The gradients were centrifuged in a Beckmann vertical rotor (type VTi-50 Beckmann Instruments, Fullerton, CA) for 3 h at 45,000 rpm, 4°C. 20 equal fractions were collected from the bottom of the tubes. Those containing the C5b-9(m) peak (fractions 3–8, corresponding to 26–38S) were pooled and used in further experiments.

Binding of Serum S-Protein to C5b-9(m) and Isolation of SC5b-9(m)

WHS was used as the source of S-protein. 35-ml aliquots of WHS were chromatographed over Sepharose 6B (4 × 90 cm) in 50 mM Tris, 100 mM NaCl, 15 mM NaN_3, pH 8.2. Flow rates were 30–40 ml/h and 18 to 20-ml fractions were collected at 4°C. Aliquots of fractions from the column were assayed by fused rocket immunoelectrophoresis with the use of specific antibodies to α_2-macroglobulin and C3 complement component (obtained from Dakopatts Immunoglobulin A/S, Copenhagen, Denmark). These two protein moieties were cleanly separated from each other by the Sepharose chromatography. Fractions commencing from the void volume and extending to cover the entire α_2-macroglobulin peak were discarded. All fractions from the C3 peak onwards were pooled, ending with the column bed volume, and concentrated to 20–30 ml (Amicon PM 10 membrane: Amicon Corp., Lexington, MA). The concentrated protein solution was then added to solutions of C5b-9(m) at a volume ratio of 1:1. The samples were dialyzed against 50 vol of isotonic saline/10 mM EDTA, pH 8.0 at 4°C for 18 h. In later experiments, EDTA was omitted with no change in the results. Thereafter, the samples were concentrated to ~8–9 ml and chromatographed over a 2- x 90-cm Sepharose 6B column in the buffer stated above. Fractions containing the SC5b-9(m) complex, initially identified by an antiserum to C5b-9(m), were pooled, concentrated to 0.5–0.8 ml and applied to a 12-ml 10–43% (wt/wt) linear sucrose density gradient containing no detergent. Centrifugation was in an SW 41 Ti rotor (Beckmann Instruments) for 16 h at 35,000 rpm, and 4°C. 20 equal fractions were collected from the bottom of the tubes. SC5b-9(m) complex was recovered in fractions 2–8 corresponding to 26–40S, and analysed by SDS PAGE, immunoelectrophoresis, and in the electron microscope.

Estimation of Sedimentation Coefficient and Molecular Radius of SC5b-9(m)

Individual fractions containing SC5b-9(m) recovered from the sucrose density gradient were dialyzed to remove sucrose, concentrated to 0.3 ml by packing the dialyzed bags in Sephadex G-100, and then recentrifuged on linear sucrose density gradients. Sedimentation coefficients were estimated from the sedimenting positions of the proteins, using serum transferrin (5.5S), IgG (7S), C3 (9.5S), IgM (19S), and SC5b-9 (22.5S) as standards, as detailed in reference 10. The protein recovered after these sedimentation analyses was then chromatographed over Sepharose 6B (1 x 60 cm) and the eluting position compared to that of SC5b-9 (molecular radius: ~11 nm) and IgM (molecular radius: ~12 nm) on the same column.

Electroimmunoassay

Rocket and fused rocket immunoelectrophoresis were performed in 1% agarose gels as previously described (1, 27). In specified cases, 0.5% (vol/vol) Triton X-100 was present in the agarose. Electrophoreses were run overnight at 2–3 V/cm.

SDS PAGE

Protein samples were made 2% in SDS and briefly boiled (5 s). A discontinuous slab gel electrophoresis system was used as in reference 8. Running and stacking gels were 10% and 5% acrylamide, respectively; both contained 6.4 M urea. Gels were stained with Coomassie Brilliant Blue.

Electron Microscopy

Negative stainings were performed as given in reference 27, using sodium silicotungstate at pH 8.2 or sodium phosphotungstate at pH 6.8 with identical

results. Sucrose was removed by dialysis before staining. Specimens were examined at 80 kV in a JEOL 100 CS electron microscope equipped with an ultra high resolution objective lens pole piece (Cs ~0.7) using apertures of 200 μm in the condensor and 40 or 60 μm in the objective. Micrographs were recorded at 50–90,000 times magnification by standard or low dose exposure techniques.

RESULTS

Fig. 1 depicts a fused rocket immunoelectrophoresis of fractions recovered from a sucrose density gradient centrifugation of DOC-solubilized, complement-treated erythrocyte membranes. The C5b-9(m) complex (arrow, Fig. 1 A), identifiable with a specific antiserum (Fig. 1 B) sediments in a broad peak covering a region of 25–40S. The high molecular weight material represents oligomeric aggregates of the complex; monodispersed C5b-9(m) cylinders are found at a region corresponding to 26–29S in this system (reference 10 and Fig. 4). The SDS PAGE pattern of C5b-9(m) as recovered from such a gradient is shown below (Fig. 3, gel a).

The rationale of the present experiments was to take isolated C5b-9(m) complexes, bind the S-protein from serum, and reisolate the ensuing SC5b-9(m) complexes. Earlier studies had indicated the molecular weight of the S-protein or its smallest
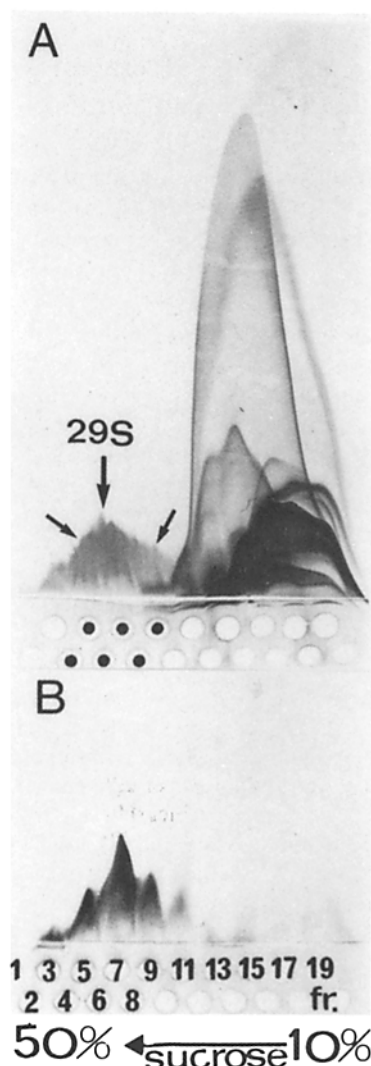

FIGURE 1 Isolation of human C5b-9(m) from target rabbit erythrocyte membranes. The membranes were solubilized in 250 mM deoxycholate and applied to linear 10–50% (wt/vol) sucrose density gradients containing 6.25 mM deoxycholate. Centrifugation was in a preparative vertical rotor as detailed in Materials and Methods. Twenty equal fractions were collected and aliquots were applied in fused rocket immunoelectrophoresis using polyspecific antibodies to human serum proteins (A) and specific anti-C5b-9(m) antiserum (B) in Triton-containing agarose gels. The C5b-9(m) complex sedimented in a broad region covering 25–40S, with a protein peak at 29S (arrows, A), and was separated from the bulk of other serum proteins. Erythrocyte membrane proteins were not identified immunochemically, but were found by SDS PAGE analyses to be located virtually quantitatively in the later gradient fractions (fraction 9 onward, results not shown). Closed circles denote the fractions that were pooled and used as starting material for the SC5b-9(m) preparation. The polypeptide gel electrophoresis pattern of C5b-9(m) thus obtained is shown in Fig. 3, gel a.

subunit to be ~88,000, as determined by SDS PAGE (16). By densitometry, three molecules of the 88,000-dalton S-protein (or subunit) appeared to become bound to each molecule of C5b-9 during formation of the fluid-phase SC5b-9 complex (16). Thus, the maximal molecular weight of the native S-protein could be assumed not to exceed 265,000 (in the case that the native protein consisted of a noncovalently linked trimer of the 88,000-mol wt subunit). The experimental approach for isolation of SC5b-9(m) took advantage of the marked difference in size between the membrane C5b-9(m) complex and the native S-protein. Human serum was first depleted of protein that eluted on Sepharose ahead of C3 (200,000 mol wt). When C5b-9(m) in detergent solution was added to the low molecular weight serum protein pool (containing S-protein) and the detergent was removed by dialysis, rechromatography immediately permitted recovery of the terminal complex in highly purified form (Fig. 2). A single centrifugation in a sucrose density gradient resulted in the recovery of the terminal complex virtually free of contaminants (Fig. 2 B). The protein was distributed over a somewhat broad region in the gradient, correponding to 26–40S, with the peak concentration at 28–30S. Recentrifugation of individual fractions from the gradient showed that this distribution was due to the presence of discrete, heterogeneous protein populations, each sedimenting true to its given position in the repeat gradient (results not shown). In no case did higher molecular sized-aggregates form, i.e. the protein did not aggregate in detergent-free solution.

In contrast, C5b-9(m) self-associated extensively in the absence of detergent to form large molecular aggregates that sedimented to the bottom of the detergent-free sucrose density gradients (not shown).

SDS PAGE of each fraction from these gradients showed the presence of C5b-C9 plus the S-protein in fractions 2–8 (40–26S). There were no conspicuous differences among the electrophoretograms from the individual fractions. Fig. 3 shows the subunit pattern of C5b-9(m) that was used as the starting material (gel a), and of the SC5b-9(m) complex recovered from the sucrose density gradient of Fig. 2 B (gel b). As reference for the migrational position of the S-protein, the pattern of fluid-phase SC5b-9 is shown (gel c). By densitometry, there are 3–4 molecules of S-protein per molecule of C6 in both SC5b-9 and SC5b-9(m). It should be noted that apart from the difference in content of S-protein, human C5b-9(m) differs from SC5b-9 in that it contains additional C9 molecules that are possibly present as disulfide-linked dimers (designated $C9_2$ in Fig. 3) (see reference 28).

Electron microscopical analyses of C5b-9(m) and SC5b-9(m) are shown in Fig. 4. Dispersed C5b-9(m) in deoxycholate solution exhibited apparent sedimentation coefficients of 26–29S and presented a hollow cylindrical structure as previously reported (Fig. 4A). Analysis of the individual SC5b-9(m) fractions from the sucrose density gradients showed that cylindrical monomers were present in fractions corresponding to 28–29S (Fig. 4B). In earlier fractions from the gradient, dimers and uninterpretable aggregates were found (Fig. 4C).

The basic structure of the C5b-9(m) complex, being a thin-walled cylinder rimmed by an annulus at one end (Fig. 4D), was unaffected by the binding of S-protein. However, additional material, appearing as a girdle at the middle of the cylinder, was consistently identified on SC5b-9(m) complexes (Fig. 4E). By inference, this material is interpreted to represent
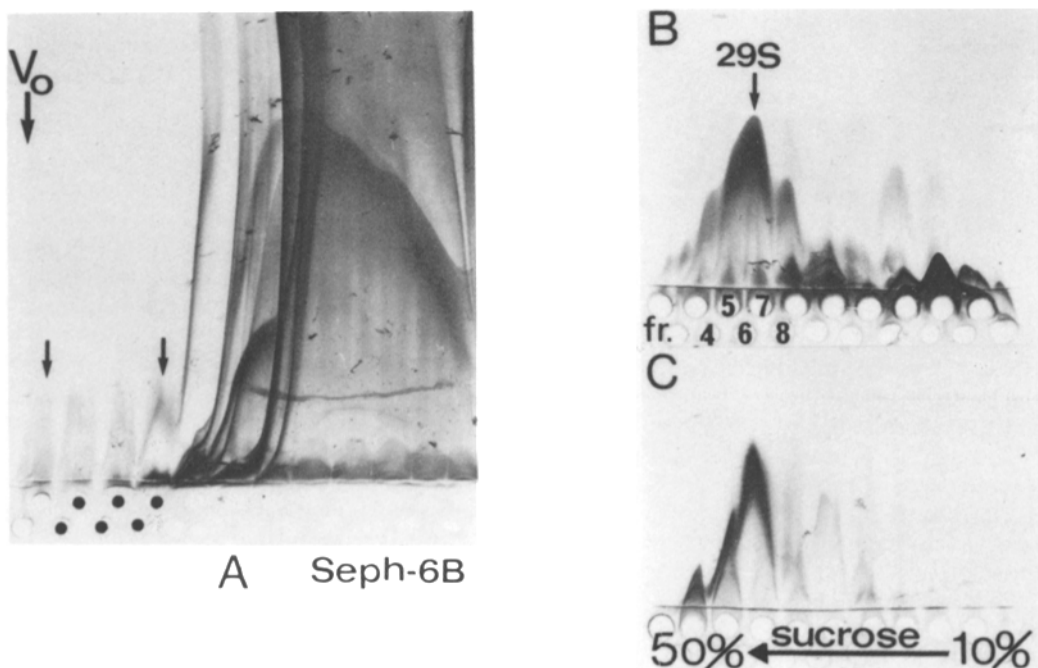

A   Seph-6B

FIGURE 2   A: C5b-9(m) recovered from the sucrose density gradient was added to EDTA-human serum that had been depleted of high molecular weight proteins (detailed in Materials and Methods). After removal of deoxycholate by dialysis, the protein sample was chromatographed over Sepharose 6B without detergent. The SC5b-9(m) complex was then recovered in early fractions from the column, (arrows), separated from the bulk of other serum proteins. Aliquots of the fractions from the column were analyzed by fused rocket immunoelectrophoresis developed with polyspecific antibodies to human plasma proteins. Closed circles denote the fractions that were pooled for subsequent sucrose density gradient centrifugation. B and C: Sucrose density gradient centrifugation of pooled fractions containing the SC5b-9(m) complex in the absence of detergent: analysis of the recovered fractions by fused rocket immunoelectrophoresis with the use of polyspecific antibodies to serum proteins (B) and specific anti-C5b-9(m) (C). The SC5b-9(m) complex was recovered as a major protein peak at 29S (arrow, B) in highly purified form.
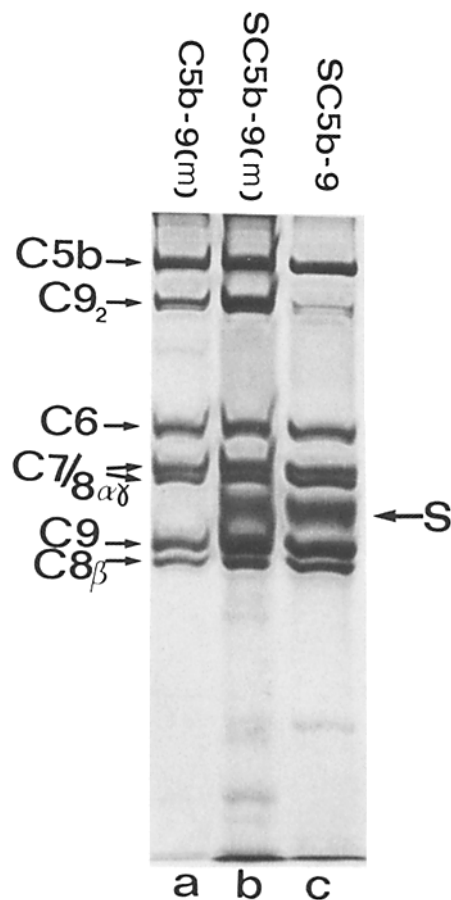
FIGURE 3   SDS PAGE of C5b-9(m) (gel a) and SC5b-9(m) (gel b); the protein samples were taken from the sucrose density gradients of Fig. 1 and 2, respectively. The individual bands have been designated according to their previous identification (references 9, 28, 29). In addition to the normal banding pattern of C5b-9(m), the SC5b-9(m) complex exhibits an additional polypeptide band (3–4 moles per complex) corresponding to the S-protein. The polypeptide composition of fluid-phase SC5b-9(m) is shown in gel c as a reference pattern for the S-protein. Note the presence of C9-dimer in C5b-9(m) and SC5b-9(m), but not in the SC5b-9(m) complex that primarily forms in the fluid-phase (see reference 28).

the bound S-protein. Whether the distribution of this material was truly rotationally symmetrical could not be determined because of shielding by the annulus in axial projections. On cylinders seen in clean, side profile, the boundary of the attached material towards the annulus was sharp, while its contour towards the originally membrane-embedded terminus of the cylinder was often blurred, probably indicating extension of material in this direction.

The 29S material was chromatographed over Sepharose 6B and the protein was found to elute in a region corresponding to that of IgM. From an assumed molecular radius of 12 nm, a sedimentation coefficient of 29S, and a partial specific volume of 0.72, a molecular weight of ~1.4 million was calculated for the SC5b-9(m) cylinder. This value is in good agreement with a C5b-9(m) molecular weight of 1–1.1 million, plus 3–4 molecules S-protein (260–350,000 daltons) per complex.

DISCUSSION

This study documents a singular case of an amphiphilic-hydrophilic molecular transition through binding of a serum protein to an amphiphilic protein complex. The serum S-protein that is responsible for this effect has earlier been implicated as an inactivator/inhibitor of nascent C5b-7 when the complex is generated in serum away from a target lipid bilayer (22, 23). The present data support this concept since they provide direct evidence for binding of the S-protein to hydrophobic surfaces on preformed, amphiphilic C5b-9(m) complexes. In the electron microscope, the S-protein then appears to be attached to the originally membrane-embedded portion of the C5b-9(m) cylinder. The collective results are fully compatible with the previously proposed C5b-9(m) model, which locates the apolar surfaces on the molecule to the thin-walled portion of the cylinder that is situated opposite the annulus (4, 6, 27).

Preparations of detergent-solubilized C5b-9(m) complexes exhibit some structural heterogeneity, although their purity by bioimmunochemical criteria is high. This may relate to a true heterogeneity of configuration arising during assembly of the complex, or it may be induced during the isolation procedure. Thus, "opened" rings (Fig. 4A and B) are quite frequent in some preparations of solubilized C5b-9(m) or SC5b-9(m), but these are hardly found on fresh, complement-lysed membranes. We consider the closed cylindrical structure, which is identical with the principle membrane lesion identified on target membranes, to be the primarily relevant structure in detergent-solubilized material.

Hydrodynamic analyses of SC5b-9(m) become possible in the absence of detergent and are consistent with a monomer nature of the complex. Dispersed C5b-9(m) complexes in detergent solution exhibit an apparent sedimentation coefficient of 26–29S and a protein molecular weight of 1–1.1 million. Each C5b-9(m) complex can bind 3–4 molecules S-protein, and the molecular weight of the resulting SC5b-9(m) complex is ~1.4 million. It should be noted that binding of the S-protein will not disrupt preexisting oligomeric aggregates of C5b-9(m). Therefore, it is necessary to ascertain by electron microscopy that the hydrodynamic data used for calculation of molecular weight apply to monodispersed protein. Since this and a previous study have shown that protein sedimenting at positions >30S (e.g. 33S as reported in references 11 and 24) is present as heterogeneous aggregates, claims to have proven a dimeric composition of C5b-9(m) based on biochemical analysis of 33S fractions (24) also cannot be accepted as valid. Indeed, if each C5b-9(m) cylinder were to represent a dimer composed of 2 molecules C5b-C8, and 12 molecules C9, the molecular weight of the complex would be 2 million, and that of SC5b-9(m) 2.5–2.7 million. Clearly, this value is not compatible with a sedimentation coefficient of 29S, or even 33–34S. In this connection, it is noteworthy that the lower molecular weight (1 million) and sedimentation coefficient (23S) of SC5b-9 formed primarily in the fluid-phase may well be due to the lower number of C9 molecules present per complex (2–3 instead of 6), as has been recognized recently (28).

Nomenclature

To distinguish among the different forms of the terminal complement complexes, we propose that the term "C5b-9(m)" rather than "MAC" (11) be used to denote the membrane complex, and "SC5b-9" be used to designate the cytolytically inactive complex that forms in serum in the absence of a target membrane. The term "SC5b-9(m)" then adequately describes the membrane C5b-9(m) complex to which S-protein has bound, as reported in this study.
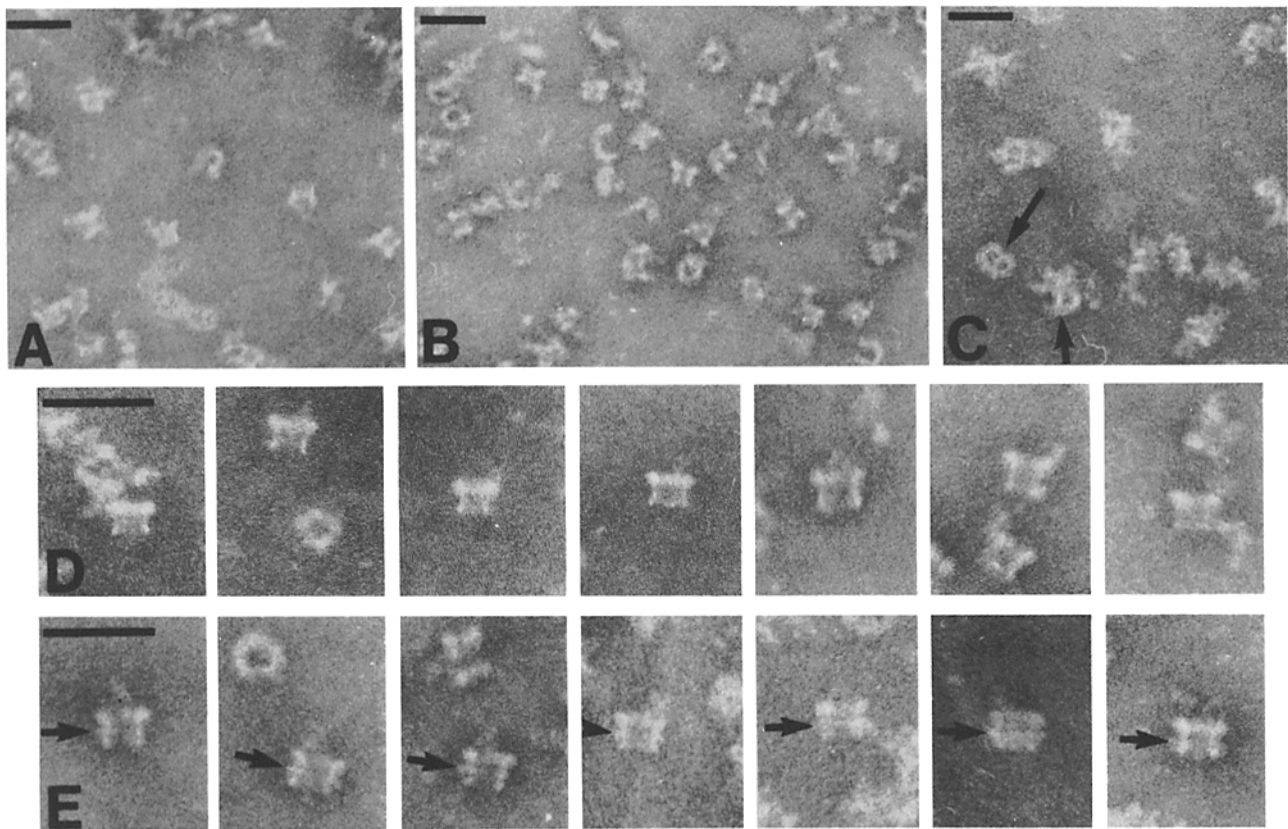
FIGURE 4 Panels A and B. Survey of 29S fractions of detergent solubilized C5b-9(m) complexes (A) and SC5b-9(m) complexes in detergent free solution (B). The majority of protein is present as cylindrically structured monomer entities. Some complexes seen in axial projections appear as opened rings in both preparations. Panel C. Survey of a 33S fraction of SC5b-9(m) complexes. Most of the material appear as heterogeneous aggregates. Some complexes are seen as "twinned" rings (arrows). Panels D and E. C5b-9(m) complexes (D) and SC5b-9(m) complexes (E) seen in side projections at high magnification. The basic structure of the molecule is that of a thinwalled cylinder rimmed by an annulus at one end. SC5b-9(m) complexes carry an additional "girdle" of material (arrows in panel E), which is interpreted to represent the S-protein. Sodium silicotungstate negative staining. All micrographs, except left four frames of panel D, were recorded with low electron exposure technique. Bars, 50 nm.

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