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Immunology

Molecular weight of the membrane C5b–9 complex of human complement: Characterization of the terminal complex as a C5b–9 monomer

(amphiphilic protein/detergent-binding/transmembrane channel)

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ABSTRACT **The hydrodynamic properties of the detergent-solubilized, terminal membrane complex of serum complement components C5–C9 [C5b–9(m)] were studied to obtain an estimate of its molecular weight. In a solution of Triton X-100/deoxycholate, the protein complex binds 17% Triton X-100 and 11% deoxycholate by weight. The sedimentation coefficient of the protein–detergent complex is 26 S as determined by sucrose density gradient ultracentrifugation, and gel filtration indicated a molecular radius of 11 nm. It was ascertained by electron microscopy that these hydrodynamic parameters apply to mono-dispersed C5b–9(m) complexes, which were observed as nonaggregated, hollow protein cylinders and were identical to the complement "lesions" formed on target membranes. The calculated molecular weight of the protein–detergent complex is approximately 1,286,300 to which the protein moiety contributes approximately 1,000,000. The results indicate that the C5b–9(m) complex formed on biological membranes is a monomer entity of the C5–C9 complement components.**

The sequential assembly of serum complement components C5–C9 into an amphiphilic C5b–9(m) complex on and in a target membrane constitutes the terminal reaction in the complement cascade that is responsible for primary membrane damage (1–5). The C5b–9(m) complex has been characterized with respect to its subunit composition (6), ultrastructure (7), detergent- and lipid-binding properties (8–10), and its capacity to generate discrete transmembrane channels (11–14). The collective data speak for the channel concept of immune cytolysis (15), which envisages C5b–9(m) as a hollow protein cylinder that forms in the lipid bilayer, creating a hydrophilic transmembrane pore through which ion leakage occurs (9, 10, 15–17).

An unresolved question pertains to the molecular composition of C5b–9(m). Initial approximations indicated a 1:1:1:1:2–3 molar ratio of the C5–C9 components (6, 18). If C5b–9(m) is a monomer of this subunit composition, a total molecular weight of approximately 800,000–850,000 would be predictable from the molecular weights of the individual complement components (19–22). Data from a recent study on the hydrodynamic properties of the complex apparently excluded a monomer composition of C5b–9(m). A molecular weight of 1,700,000 was calculated for the complex in deoxycholate solution (18). This led to the conclusion that C5b–9(m) is a dimer of C5b–9—i.e., $(C5b–9)_2$ (18).

Because the C5b–9(m) complex is an amphiphilic, detergent-binding macromolecule, any molecular weight estimate based on conventional hydrodynamic data must take into account the amount of protein-bound detergent (23–25). Moreover, because the complex exhibits a tendency to aggregate even in detergent solution (7), it is necessary to ascertain that gel filtration and sedimentation data apply to the protein in its non-aggregated state. Because these factors were not considered (18), we reinvestigated the physicochemical properties of detergent-solubilized C5b–9(m). We present data showing that the protein complex assembled on a target membrane has a

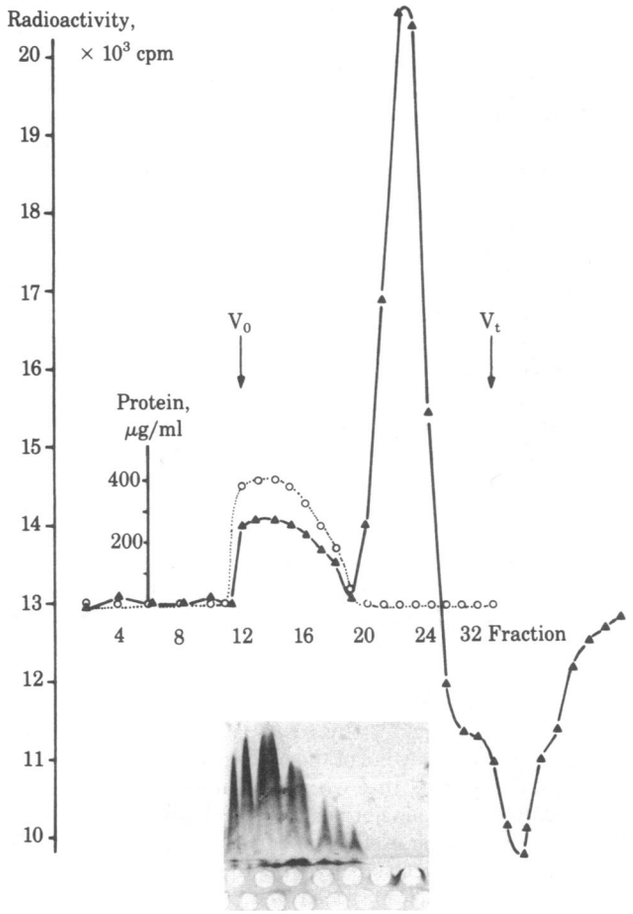

FIG. 1. Gel filtration of C5b–9(m) in the presence of 1.2 mM Triton X-100 and 1.25 mM [^{3}H]deoxycholate. Column: Sepharose 6B, 1.0 × 60 cm. The fused rocket immunoelectrophoresis shows the elution profile of C5b–9(m), detected with polyspecific antibodies to human serum proteins. The fractions were assayed for protein (○) and ^{3}H (▲). V_0 and V_t denote the void elution volume and the included (bed) volume of the column, respectively.

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Abbreviations: C, complement; C5b–9(m), membrane C5b–9 complex of the C5b–C9 complement components formed in a lipid bilayer; SC5b–9, fluid-phase C5b–9 complement complex.

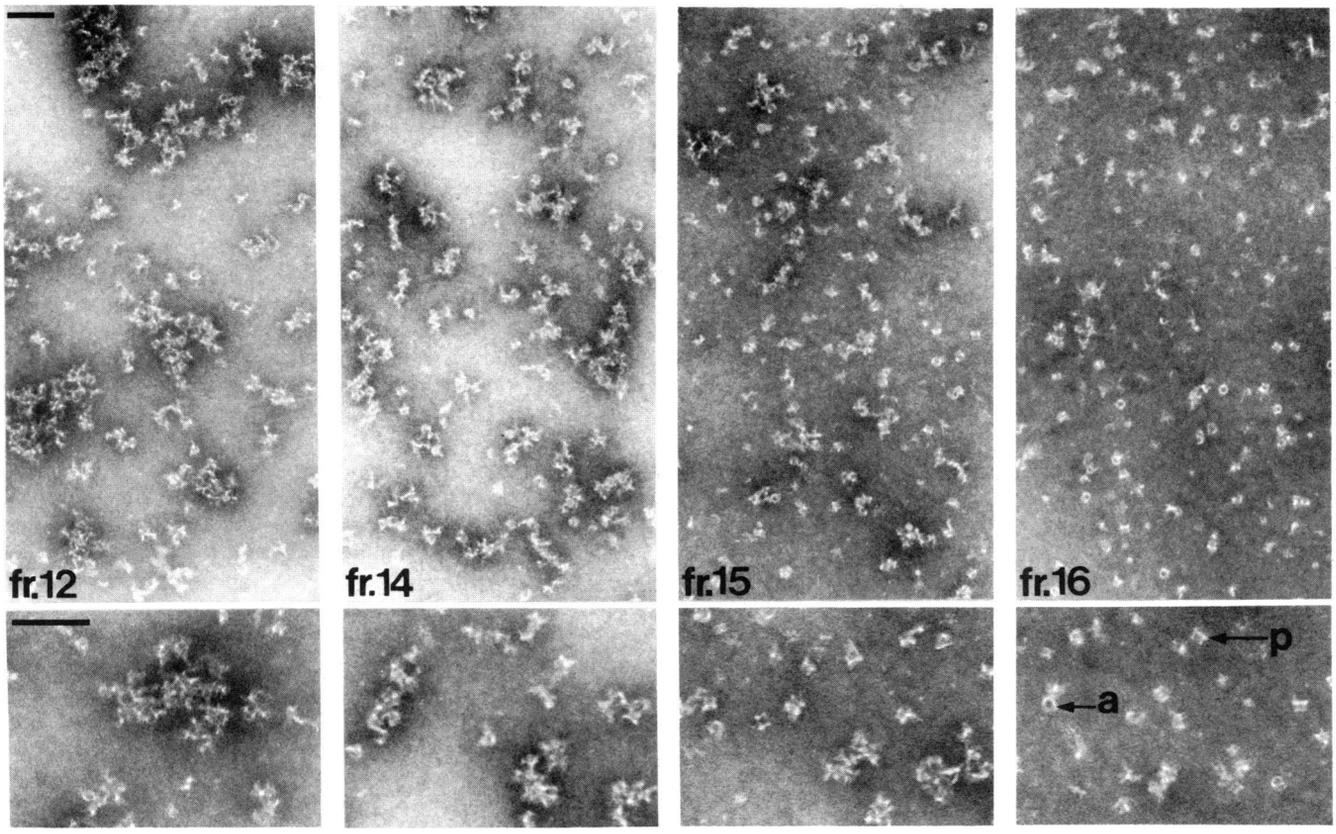

FIG. 2. Electron micrographs of C5b–9(m)-containing fractions from the Sepharose 6B column of Fig. 1. Fraction (Fr.) 16, corresponding to Kav 0.25, contained predominantly dispersed C5b–9(m) cylinders, which can be seen in profile (p) and axial projection (a). Earlier fractions (15, 14, 12) contain C5b–9(m) in increasingly aggregated states. Negative stain: sodium silicotungstate. Scale bars: 100 nm.

molecular weight of approximately 1,000,000 and, therefore, represents a monomer entity and not a dimer of the C5–C9 complement components.

MATERIALS AND METHODS

Biochemicals were from Serva (Heidelberg) and Merck (Darmstadt). [^{3}H]Deoxycholic acid (specific activity, 4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [^{3}H]Triton X-100 (specific activity, 1.58 mCi/mg) were from New England Nuclear. Sepharose 4B and 6B were obtained from Pharmacia (Uppsala). All antisera were purchased from Dakopatts (Copenhagen) with the exception of anti-C5b–9(m), which was produced as described (8).

Experimental Procedures. Complement-lysed, sheep erythrocyte membranes (30 ml) prepared exactly as described (7) were solubilized in 1% Triton X-100, and a clear extract was recovered through centrifugation. The detergent extract was chromatographed on a Sepharose 6B column (4.5-cm diameter, 90-cm height) equilibrated with 10 mM Tris/50 mM NaCl/15 mM NaN_3, pH 8.2, containing 2.4 mM Triton X-100 and 2.5 mM deoxycholate at a flow rate of 45 ml/hr at 4°C, and 22-ml fractions were collected. Those containing C5b–9(m) were pooled, supplemented with 2.4 mM Triton X-100 and 2.5 mM deoxycholate, and concentrated over Amicon Diaflo PM 30 membranes to ≈4–5 ml. For isolation of native C5b–9(m), samples were immediately rechromatographed over a second Sepharose 6B column (2-cm diameter, 90-cm height) in the same buffer (flow rate, 16 ml/hr; fractions, 8 ml). For isolation of proteolyzed C5b–9(m) (7–9), samples were treated with a combination of trypsin and α-chymotrypsin at individual enzyme concentrations of 0.1 mg/ml for at least 3 hr at 37°C, and then they were similarly chromatographed. After rechromatography, proteolyzed C5b–9(m) was recovered in virtually pure form (7), whereas native C5b–9(m) was contaminated with small amounts of several serum proteins, including high molecular weight forms of membrane-derived C3 and C4. Native and proteolyzed C5b–9(m) were eluted at exactly the same positions on the Sepharose columns (7). Fractions containing the complex were pooled and concentrated to approximately 1 ml. The final protein concentrations ranged from 2.5 to 4.0 mg/ml. $NaDodSO_4$/polyacrylamide gel electrophoresis produced the polypeptide patterns for native and proteolyzed C5b–9(m) as detailed (26).

The amount of detergent bound to C5b–9(m) was determined by chromatographing 1 ml of purified C5b–9(m) (2.5–4.0 mg) over a Sepharose 6B column (1-cm diameter, 60-cm height) equilibrated with the same Tris/NaCl buffer containing 1.2 mM Triton X-100 and 1.25 mM deoxycholate. Either radioactive Triton or deoxycholate was added to the sample and buffer to give between 35,000 and 75,000 cpm/ml. The columns were eluted at 3.2 ml/hr and 22°C, and 1.6-ml fractions were collected. Each fraction was assayed for protein and analyzed immunoelectrophoretically; radioactivity in 200-μl aliquots was determined 16 hr after addition of 1 ml of Soluene 350 (Packard) and 10 ml of scintillator fluid (Rotiszint 22, Roth Labs, Heidelberg). The amount of protein-bound detergent was calculated by correlating the increase of radioactivity to the amount of protein in the respective fractions as described (27).

For estimation of sedimentation coefficients, 300-μl aliquots of each C5b–9(m)-containing fraction from the detergent-binding experiments were applied to linear 10–43% (wt/wt) sucrose density gradients (total volume, 12 ml) prepared in the same Tris/NaCl buffer containing 2.4 mM Triton X-100 and 2.5 mM deoxycholate. Centrifugation was performed at 35,000 rpm in an SW 41 Ti rotor (Beckman ultracentrifuge L2 65B) for 16 hr at 4°C. Aliquots (300 μl) of human serum diluted 1:1 with saline

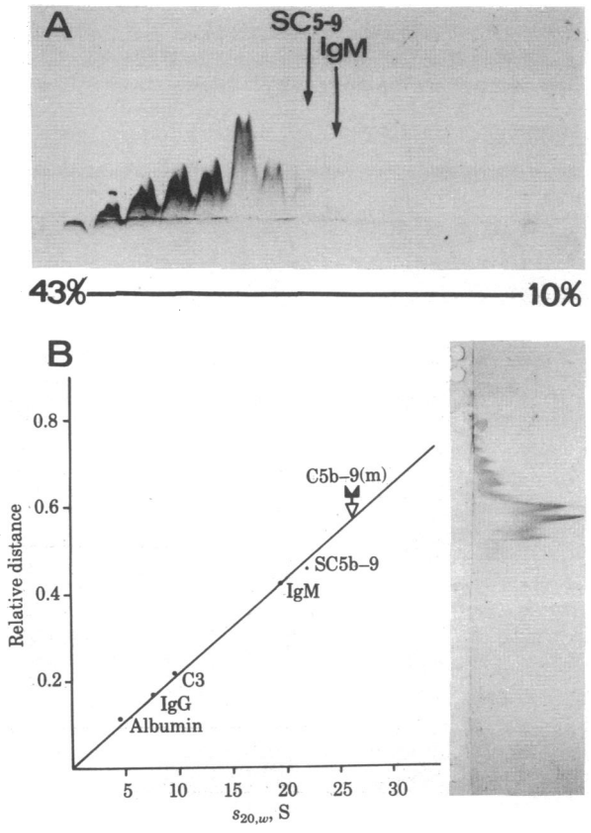

FIG. 3. (*A*) Distribution of C5b–9(m) contained in an early Sepharose 6B fraction (Fig. 1, fraction 13) in a sucrose density gradient after centrifugation at 35,000 rpm for 16 hr at 4°C (rotor SW 41Ti). The sedimentation positions of IgM and SC5b–9 in identical gradients are shown. C5b–9(m) complexes are distributed over a wide range corresponding to 25–40 S. The rocket immunoelectrophoresis was performed by using specific anti-C5b–9(m) antiserum. (*B*) Distribution of dispersed C5b–9(m) complexes (Fig. 1, fraction 16) in a parallel gradient. A symmetrical C5b–9(m) peak is obtained with $s = 26$ S. The plot depicts the s of marker proteins in an identical gradient.

and 300 μl of a preparation of SC5b–9 (28) were applied to parallel, identical gradients. After centrifugation, 16 or 20 equal fractions were collected from the bottom of the tubes, and the sedimentation positions of serum albumin (4.5 S), IgG (7 S), C3 (9.5 S), IgM (19 S), and SC5b–9 (22.5 S) were established by rocket immunoelectrophoresis with specific antibodies. A linear plot of $s_{20,w}$ versus relative sedimentation distance was obtained, and from this the sedimentation coefficient of C5b–9(m) was determined (29, 30).

For estimation of molecular radius, 1-ml aliquots of individual fractions containing C5b–9(m) from the Sepharose 6B columns were rechromatographed over a Sepharose 4B column (1-cm diameter, 58-cm height) in the same buffer system. The elution position of C5b–9(m) was determined by rocket immunoelectrophoresis and electron microscopy and was compared with that of SC5b–9 on the same column. SC5b–9 runs were done without detergent in the buffer.

Electron microscopy and quantitative immunoelectrophoresis were performed as described (7, 9). Protein in fractions from the Sepharose columns was quantified by the method of Lowry *et al.* (31) with 0.4% $NaDodSO_4$ present in the samples. Initial calibrations were established by parallel amino acid analyses. Native C5b–9(m) was further quantified by rocket immunoelectrophoresis (32).

One set of experiments was performed with a sample of highly purified, native C5b–9(m) recovered after detergent-solubilization of reconstituted liposomes with the same results.

RESULTS

Binding of Triton X-100 and Deoxycholate to C5b–9(m). The fused rocket immunoelectrophoresis (Fig. 1) depicts the elution profile of purified C5b–9(m) on a Sepharose 6B column in the presence of Triton and deoxycholate. The complex was eluted in a somewhat broad peak, beginning directly with the column void volume and extending into a region of Kav ≈0.3. Upon chromatography in the presence of a radioactive detergent, a detergent peak was coeluted with the protein (Fig. 1). This detergent peak was absent in control and blank runs (not shown) and was well separated from the large peak appearing at Kav ≈0.65 that corresponds to the free detergent micelles (27). From the measured increase of detergent relative to the protein

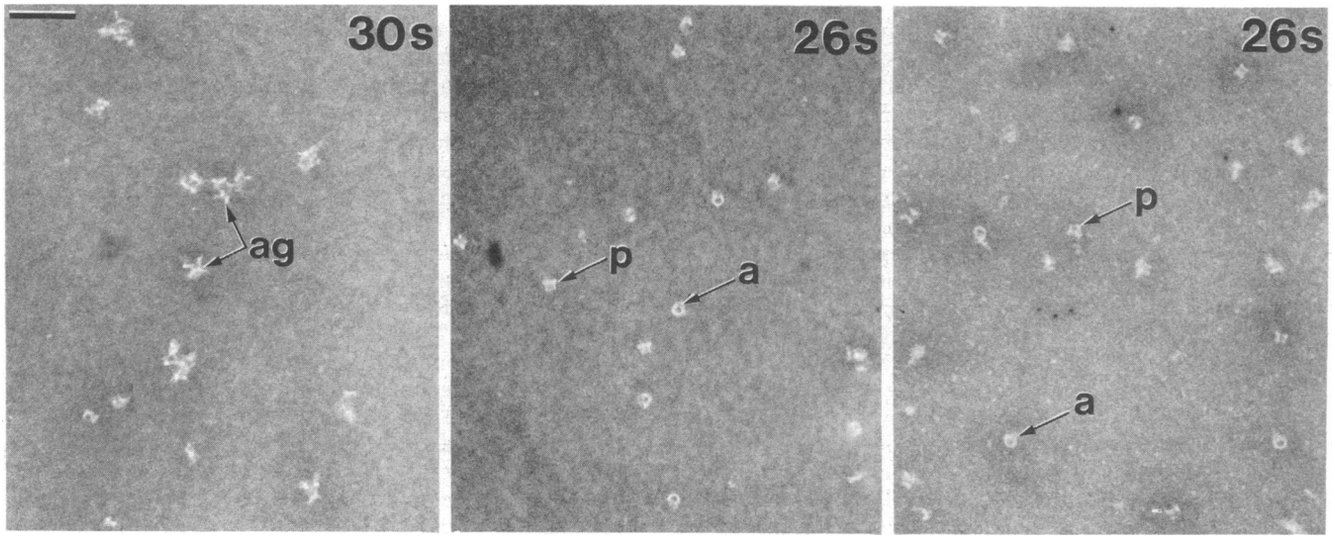

FIG. 4. Electron micrographs of sucrose density gradient fractions containing C5b–9(m) sedimenting at 26 S and 30 S. The latter contain C5b–9(m) in aggregated form (ag), whereas the 26S fractions exclusively contain monomer C5b–9(m) cylinders (axial a; profile, p). (*Left* and *Center*) Preparation of proteolyzed C5b–9(m). (*Right*) Preparation of native C5b–9(m), which carries some contaminants. Negative stain: sodium silicotungstate. Scale bars indicate 100 nm.

content in each C5b–9(m)-containing fraction, the amount of detergent bound to C5b–9(m) was determined individually for Triton and deoxycholate, and values of binding of 17% (wt/wt) detergent and 11% (wt/wt) protein were obtained. The degree of binding was equivalent for both native and proteolyzed C5b–9(m), and no conspicuous differences of binding ratios were observed among the individual fractions from the column.

The set of electron micrographs in Fig. 2 shows the state of dispersion of the C5b–9(m) complexes in the individual fractions from the same column. Those fractions that were eluted with the void volume contained aggregated complexes. Less aggregated material and truly dispersed C5b–9(m) complexes appeared in the following fractions, and the peak of nonaggregated complexes appeared at a Kav of approximately 0.25. Monomer complexes accounted for 20–25% of the total protein. They were visualized as ring or rectangular structures identical to C5b–9(m) complexes on target membranes (7).

Sedimentation Coefficient and Molecular Radius. When C5b–9(m) contained in an early fraction from the Sepharose column was centrifuged in a sucrose density gradient, a wide distribution of protein was discerned ranging from 25 to 40 S (Fig. 3*A*). However, a late fraction from the same column (Kav 0.25) contained material sedimenting in a symmetrical peak. The sedimentation coefficient *s* calculated for these complexes was 26 ± 1 S in six individual experiments, identical results being obtained for both native and proteolyzed C5b–9(m) (Fig. 3*B*). Fig. 4 shows electron micrographs of 26S and 30S fractions from such sucrose density gradients. Fractions corresponding to >28 S always contained C5b–9(m) in an aggregated state, whereas the 26S fractions contained perfectly intact, dispersed C5b–9(m) cylinders.

Aliquots of C5b–9(m) recovered from the Sepharose 6B columns were rechromatographed over Sepharose 4B. Material contained in early fractions from the Sepharose 6B columns representing aggregated C5b–9(m) were eluted asymmetrically ahead of SC5b–9 (not shown). However, dispersed C5b–9(m) complexes in a late fraction from the Sepharose 6B column were eluted in a symmetrical peak with a Kav of 0.33–0.40. Electron micrographs revealed that the fractions with elution characteristics identical to those of SC5b–9 (Kav 0.4) contained nonaggregated C5b–9(m) (Fig. 5, fraction 27). Fractions ahead of this region contained increasing amounts of dimer and trimer aggregates (e.g., Fig. 5, fraction 24) that had formed during rechromatography. Thus, the molecular radius of C5b–9(m) is taken to be equivalent to that of SC5b–9. The molecular radius of SC5b–9 calculated from its molecular weight of 1,000,000 and a sedimentation coefficient of 22.5 S is approximately 11 nm. This value agrees with the diffusion coefficient of SC5b–9 given previously (18).

Molecular Weight of C5b–9(m). The partial specific volume of the protein complex was calculated from its amino acid composition (33) and found to be 0.72. The partial specific volume of the protein–detergent complex as calculated from the amount of bound detergent and the individual partial specific volumes of the detergents (Triton X-100: 0.908; deoxycholate: 0.778; ref. 23) was 0.75. By applying the obtained values of 26 S and 11-nm molecular radius, a molecular weight of 1,286,300 for the protein–detergent complex is obtained by conventional calculations (23, 30). By subtracting the weight of bound detergent (23), the molecular weight of the protein moiety is determined to be approximately 1,000,000.

DISCUSSION

Our data show that the C5b–9(m) complex assembled on biological membranes has a molecular weight of approximately 1,000,000 and, thus, probably represents a monomer entity of the C5–C9 components. This conclusion is at variance with a previous report, in which C5b–9(m) was stated to be a dimerized complex. Two sources of error probably explain this divergence. First, because the complex tends to aggregate even in detergent solution, it is necessary to ascertain that the physicochemical data obtained do relate to nonaggregated material. In our experience, material exhibiting a *S* >28 S in a sucrose density gradient or eluting ahead of SC5b–9 on an agarose column is aggregated. The cause for this aggregation, which we

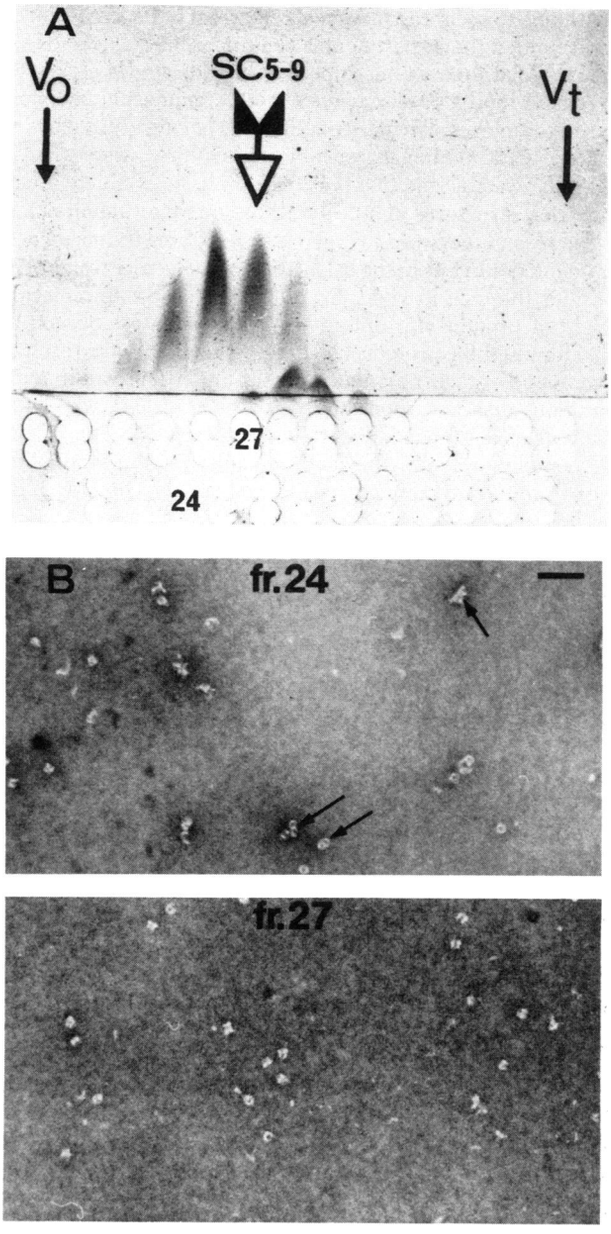

FIG. 5. (*A*) Elution of dispersed C5b–9(m) (Fig. 1, fraction 16) upon rechromatography on Sepharose 4B. The void elution volume (V_o), the bed volume of the Sepharose 4B column (V_t), and the position of SC5b–9 in the eluate (peak fraction) are indicated. The fused rocket immunoelectrophoresis was developed with a polyspecific antiserum to human serum proteins. (*B*) Electron micrographs of fractions containing C5b–9(m) from the same Sepharose 4B column. Fraction 27 corresponding to Kav 0.4 contains monodispersed C5b–9(m) cylinders. Earlier fractions (e.g., fraction 24) contain increasing amounts of dimer and trimer aggregates of C5b–9(m) (arrows). Negative stain: sodium silicotungstate. Scale bar: 100 nm.

have found to occur to a more marked extent in 1% deoxycholate alone (under the experimental conditions in ref. 18), is unknown. Polar interactions appear to be indicated, because the aggregates bind a similar amount of detergent as the monodispersed complexes. We place emphasis on having utilized hydrodynamic data for nonaggregated C5b–9(m) to determine its molecular weight, and that the 26S complexes exhibited intact, cylindrical structures identical to those on target membranes.

The second source of divergence derives from the corrections for bound detergent in the calculation of molecular weight. We consider the data to yield primarily the composite molecular weight of protein *plus* detergent, as has been established for other amphiphilic proteins (23). The correction factor resulting from bound detergent is much higher under our experimental conditions than that reported by Podack *et al.* (34), who found a binding of only 86 mol deoxycholate to each C5b–9(m) monomer complex. Possibly this low value of detergent binding derived from the severely aggregated state of the complex in deoxycholate alone.

A molecular weight of approximately 1,000,000 would be in good agreement with the ultrastructural data on the complex (7, 9, 35) and dictates that the macromolecule is truly hollow, because the molecular weight of a compact protein globule of the same dimensions (molecular radius, 11 nm) would be several times higher. The value of 1,000,000 is still higher than the sum of the individual molecular weights of C5–C9 components in a monomer complex. Several possible reasons for this discrepancy are apparent. Molecular weight estimations by the present method can only yield approximations. Determination at sedimentation equilibrium would be desirable, but in our hands this has not yet been possible because of aggregation of C5b–9(m) during centrifugation. The molar ratio of 1:1:1:1:2–3 for C5b–C9 in the complex has only been inferred through densitometric approximations of gel electrophoretograms, and may require reexamination. The molecular weights of the individual C6–C9 components also are not unequivocally established. These minor uncertainties do not alter the basic recognition that the membrane lesion of complement is created through association of a C5b–9 monomer in a lipid bilayer, resulting in the generation of a transmembrane channel that is probably walled by the hollow, inserted protein molecule.

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