

THE MEMBRANE ATTACK MECHANISM OF COMPLEMENT

Isolation and Subunit Composition of the C5b-9 Complex*

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The membrane attack complex of complement (C) represents a multimolecular assembly which involves C5, C6, C7, C8, and C9 (1, 2). The complex constitutes the only known mechanism of blood plasma which is capable of impairing biological membranes. A comprehensive study was therefore initiated with the aim to elucidate its composition, structure, and the manner in which it imparts its cytolytic function.

In this communication the isolation and dissociation of the complex is reported. It was found that each of the five terminal components is contained in the complex as isolated from activated whole human serum. Whereas C5 is incorporated in a form which is physicochemically distinct from its nonactivated precursor, such differences could not be detected for C6, C7, C8, or C9. Multiple molecules of C9 were bound to the complex, but only one molecule of each of the other proteins was present. In addition, an unidentified serum protein was found to be an integral part of the complex. Its function and identity is under study.

Materials and Methods

Human C Components and C Reagents. Highly purified C5 (3), C6 (4), and C7 (5) were obtained according to previously published methods. Highly purified C8 and C9 were prepared from the same pool of human serum by modification of the previously published methods (6, 7). These modifications will be presented in detail elsewhere.¹

C components were radiolabeled with either ¹²⁵I or ¹³¹I by the chloramine-T method of McConahey and Dixon (8). After labeling, each component was dialyzed for 48 h at 4°C, against 2 × 10 liters of 0.15 M NaCl containing 50 mM chloramphenicol (donated by Parke, Davis & Co., Detroit, Mich) and 25 μM kanamycin sulfate (Bristol Laboratories Div., Bristol-Meyers Co., Syracuse, N. Y.). The specific radioactivities of the preparations used were 4 × 10⁵-3 × 10⁶ cpm/μg.

Preparation of Sheep Erythrocyte-Antibody-C Complexes. EAC1,4^{xy}2, EAC1-7, and EAC1-8 (1) were prepared as previously described.

Isolation of the C5b-9 Complex. 200-250 ml of fresh human serum, containing 50-200 μg of

*This is publication number 909 from the Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif. This work was supported by National Institutes of Health Program Project Grant AI 07007 and a Program Project Grant from the National Heart and Lung Institute, HL-16411.

‡ Recipient of a Research Career Development Award 1-KO4-CA70841-01 from the U. S. Public Health Service.

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¹ Kolb, W. P. and H. J. Müller-Eberhard. Manuscript in preparation.

[¹²⁵I]C5, -C6, -C7, -C8, or -C9, was incubated with 5 g of particulate inulin (Pfanstiehl Labs., Inc., Waukegan, Ill.) at 37°C for 90–120 min with frequent mixing. Inulin was removed by centrifugation at 9,000 g for 30 min and the supernate was subjected to ascending column chromatography on a Biogel A-15 M column (5 × 90 cm) (Bio-Rad Laboratories, Richmond, Calif.). The column had been equilibrated and was eluted with veronal buffer containing 1 mg of sodium azide/ml. The flow rate was 15–20 ml/h and 6 ml fractions were collected. Column fractions were assayed for protein by the method of Lowry (9), ¹²⁵I radioactivity, and the distribution of several serum proteins by immunochemical analysis. Fractions containing the C5b-9 complex, as measured by incorporation of a ¹²⁵I-radiolabeled terminal component (see Fig. 1), were pooled and concentrated by ultrafiltration to a vol of 5–7 ml. This pool was applied 7.5 cm from the cathode of a 1 × 15 × 50-cm block of Pevikon in barbital buffer, pH 8.6, ionic strength 0.05. After electrophoresis at 300 V for 40 h and 4°C, the block was cut into 1.25-cm segments and eluted twice with saline. The fractions were assayed for protein, ¹²⁵I, and inhibition of fluid phase C9 hemolytic activity by the C5b-9 complex as previously described (2). The C5b-9 pool was concentrated to 2 ml, and 0.5 g of CsCl was added to obtain a density of 1.2. This solution was centrifuged in a Beckman 40 rotor at 35,000 rpm for 3 h at 4°C using an adaptor for 2-ml cellulose nitrate tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The tube was punctured and the bottom two-thirds of the CsCl solution was collected and dialyzed against 10 liters of phosphate-buffered saline (PBS) for 16 h at 4°C.

Analytical Ultracentrifugation. The C5b-9 preparations, before and after removal of lipoproteins, were examined by analytical ultracentrifugation employing a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Spinco Div.) with schlieren optics. Either plain or 2° wedge windows, double sector cells containing 0.45 ml of sample, and 0.45 ml PBS were used in an AN-D rotor at 40,000 or 52,000 rpm and 20°C.

Cellulose Acetate Electrophoresis. Cellulose acetate strip electrophoresis was conducted using a Beckman microzone electrophoresis apparatus, with barbital buffer, pH 8.6, ionic strength 0.075, at a constant voltage of 250 V for 20 min. Protein bands were visualized by staining with 0.5% Amido Black and destaining with 5% acetic acid.

Antisera. Antisera against whole human serum, IgM, IgG, α₂-macroglobulin, and β-lipoprotein were prepared in rabbits and obtained from Behring Diagnostics, Somerville, N. J. Antisera against C3, C5, C6, C7, C8, and C9 were prepared in rabbits by injection into the popliteal lymph nodes according to Goudie et al. (10). For C3, C7, C8, and C9, the respective protein band (50–100 μg) was sliced from polyacrylamide disc electrophoresis gels and mixed with an equal volume of complete Freund's adjuvant for injection.

Dissociation of the C5b-9 Complex. Isolated C5b-9, radiolabeled with [¹²⁵I]C5, -C6, -C7, -C8, or -C9 was incubated with a variety of reagents in a final vol of 200 μl at 37°C for 14–24 h. The following reagents were employed: 0.1–1.0% sodium dodecyl sulfate (SDS); 8 M urea; 200 mM EDTA; 2 M potassium iodide; 2 M Tris buffer, pH 10.9; 1 M acetic acid, pH 3.5; 90% ethanol; 5% sodium desoxycholate; and chloroform-methanol (1:1). The samples, containing ¹³¹I-labeled bovine serum albumin (¹³¹I]BSA),² ¹³¹I-labeled human gamma globulin (¹³¹I]HgG), and [¹³¹I]thyroglobulin as reference substances, were subjected to analysis on 10–40% linear sucrose density gradients in veronal buffer, pH 7.4, 0.15 M NaCl, using a Beckman SW 50.1 (Beckman Instruments, Inc., Spinco Div.) rotor for 16 h at 35,000 rpm and 4°C. 10-drop fractions were collected by puncturing the bottom of the tubes and the ¹²⁵I and ¹³¹I radioactivity was determined using a two channel Nuclear Chicago automatic gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

SDS-Polyacrylamide Gel Electrophoresis. 40–200 μg of highly purified C5b-9 containing [¹²⁵I]C5, -C6, -C7, -C8, or -C9 were incubated in a final vol of 25–200 μl of 0.1–1.0% SDS at 37°C for 16–24 h. [¹³¹I]HgG, [¹³¹I]BSA and [¹³¹I]ovalbumin were added as internal molecular weight markers. Before electrophoresis, 10–50 μl of glycerol and 2–10 μl of 2% bromophenol blue were added to each sample. Electrophoresis was conducted using the continuous phosphate-buffered system of Weber and Osborn (11). Briefly, 5–9% gels were made with 0.09 M phosphate buffer, pH 7.35 and 0.2% SDS. The upper and lower electrode vessels contained a 1:2 dilution of this buffer. Electrophoresis was conducted at a constant current of 8 mA/gel until the bromophenol blue tracking dye had traveled to

² Abbreviations used in this paper: BSA, bovine serum albumin; HgG, human gamma globulin; KAF, conglutinin-activating factor; PBS, phosphate-buffered saline, 0.05 M phosphate, pH 7.5, 0.15 M NaCl, 50 mM cloramphenicol; SDS, sodium dodecyl sulfate; WHS, whole human serum.

within 1 cm from the bottom of the gel. Analytical gels were either 5×70 mm or 6×125 mm. Gels were stained with 0.02% Coomassie Brilliant Blue (K & K Laboratories, Inc., Plainview, N. Y.) in 10% TCA and destained electrophoretically in 7.5% acetic acid. Stained protein bands were marked with copper wire and the gels sliced into 2-mm segments and ^{125}I and ^{131}I radioactivity determined.

Molar Ratios. 100 μg of C5b-9 was examined by analytical SDS-polyacrylamide gel electrophoresis as described above. After destaining, the gels were scanned at 600 nm employing a Gilford model 2000 recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Beckman DU spectrophotometer (Beckman Instruments, Inc., Spinco Div.) equipped with a linear transport attachment. A chart speed setting of 8 was used, the absorbance limits were set from 0-0.1, and the gels were scanned at 0.5 cm/min. Tracings of the individual protein zones were made on graph paper, which were cut out and weighed. The area of each peak, i.e. the weight of each tracing, was presumed to be proportional to the amount of each component protein present in the complex. Relative moles of each protein were calculated by dividing the weight of each individual area by the molecular weight of the corresponding protein. Molar ratios were expressed relative to C7 because this peak was completely separated from the others.

Results

Isolation of the C5b-9 Complex. Examination of the membrane attack complex of C necessitated its isolation. The C5b-9 complex was formed in fresh human serum, containing ^{125}I C8, by activation of C with particulate inulin. The supernate was subjected to ascending column chromatography employing Biogel A-15 M. As seen in Fig. 1, C8 radioactivity had a bimodal distribution. C8 incorporated into the C5b-9 complex eluted with IgM and β -lipoprotein, and free C8 eluted with IgG. The fractions were pooled as indicated (fractions 55-75), concentrated, and subjected to Pevikon block electrophoresis as seen in Fig. 2. The C5b-9 complex, as detected by incorporated C8 radioactivity and inhibition of fluid phase C9 hemolytic activity (2), migrated as an α -globulin and was well separated from the slower migrating IgM, but not from the antigenically defined β -lipoprotein. The latter invariably was partially associated with the C5b-9

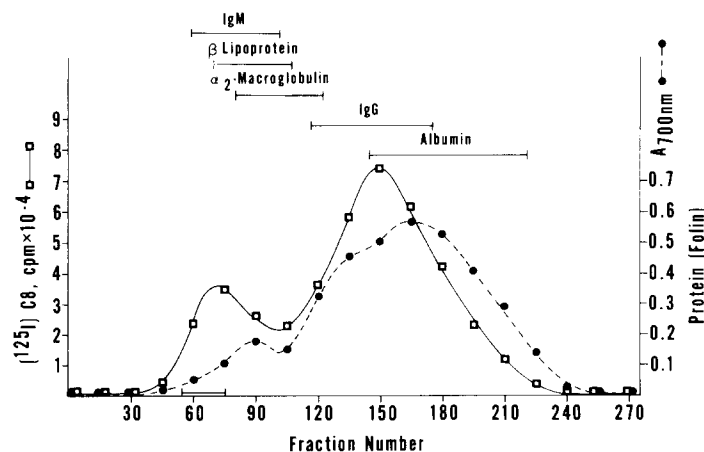


FIG. 1. Molecular sieve column chromatography as the first step in the purification of the C5b-9 complex. WHS containing ^{125}I C8 was activated with particulate inulin and subjected to chromatography on Biogel A-15 M (5×90 -cm column). The elution of radiolabeled C8 is seen relative to that of several serum proteins. Fractions indicated by the bar near the abscissa were pooled for preparative electrophoresis.

complex and it was also found as a distinct protein peak in the β -globulin region. Removal of the low density β -lipoprotein from the Pevikon block pool was achieved by flotation in CsCl. Table I summarizes the isolation of the C5b-9 complex, and Fig. 3 shows an electrophoretic examination of each protein pool obtained during the isolation procedure. C5b-9 preparations were also examined by analytical ultracentrifugation as seen in Fig. 4, which shows the schlieren patterns of three different preparations, one before and two after removal of low density lipoprotein. The observed sedimentation coefficient of the C5b-9 complex was inversely proportional to its concentration and ranged from 21.6S to 26.7S (Fig. 5). The corrected sedimentation coefficient, $s_{20,w}$, was determined to be 28.5S and the concentration dependence of the sedimentation rate was 1.1S/mg/ml.

Immunochemical Characterization of the C5b-9 Complex. The ability of the complex to react with monospecific rabbit antisera to the various terminal C components was investigated by Ouchterlony test. Fig. 6 demonstrates that antisera to C5, C6, C7, C8, and C9 are able to recognize the C5b-9 complex.

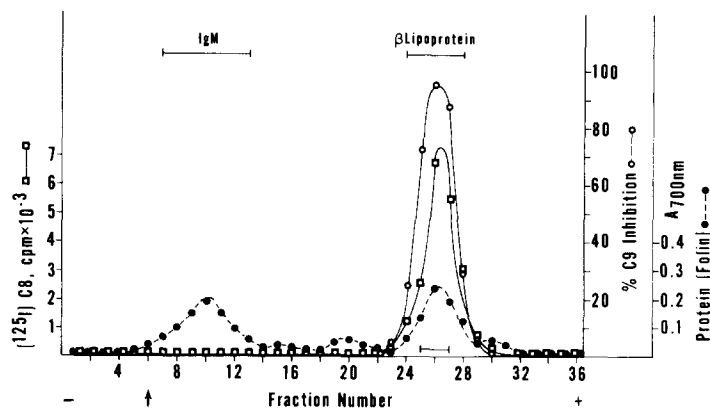


FIG. 2. Pevikon block electrophoresis as the second step in the purification of the C5b-9 complex. The Biogel A-15 M pool was concentrated and applied, as indicated by the arrow, to a 1 x 15 x 50-cm block of Pevikon in barbital buffer, pH 8.6, ionic strength 0.05. Electrophoresis was conducted at 300 V for 40 h at 4°C. The C5b-9 complex had the mobility of an α -globulin. Fractions were pooled as indicated by the bar near the abscissa.

TABLE I
Isolation of the C5b-9 Complex from Human Serum

Step	Procedure
1. C5b-9 complex formation:	Activation by inulin of 200-250 ml fresh serum containing [125 I]C5, -C6, -C7, -C8, or -C9 at 37°C for 90-120 min.
2. Chromatography on Biogel A-15 M:	Veronal-buffered saline, pH 7.5.
3. Pevikon block electrophoresis:	Barbital buffer, pH 8.6, ionic strength 0.05, 40 h, 300 V, 4°C.
4. Flotation of β -lipoprotein in CsCl:	25% (wt/wt) CsCl, density 1.2, centrifugation 35,000 rpm, 3 h, 4°C.
5. Removal of CsCl:	Dialysis against PBS-containing antibiotics.

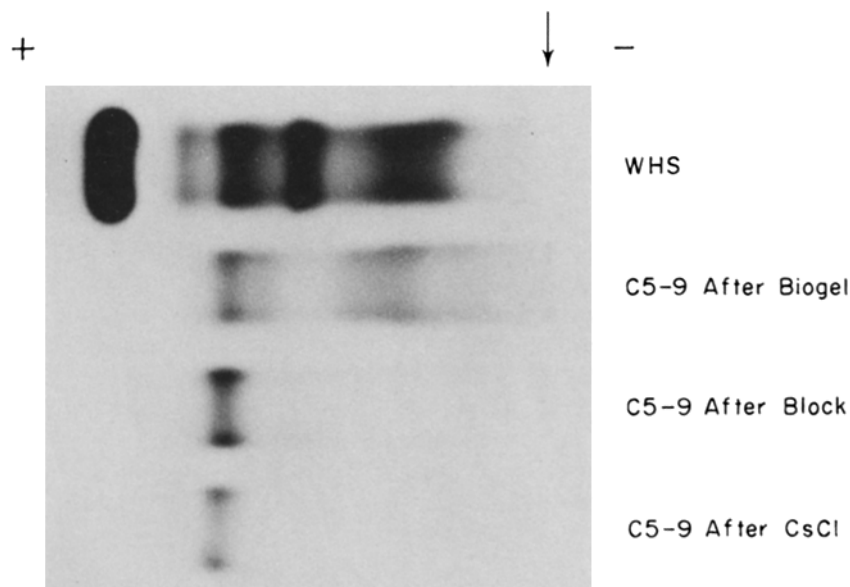


FIG. 3. Cellulose acetate strip electrophoresis of the C5b-9 complex containing pools obtained after each purification step. The arrow indicates the point of application and the cathode is to the right. Electrophoresis was conducted employing buffered barbital, pH 8.6, ionic strength 0.075, at 250 V for 20 min.

Whereas these antisera give a pattern of nonidentity when reacted with nonactivated human serum, they produce a single precipitin ring with isolated C5b-9 complex, indicating that all antibodies are reacting with the same molecular entity. Both patterns shown in Fig. 6 were produced with comparable amounts of antigen. The C5b-9 complex was employed at a concentration of 1 mg/ml. Since C6 represents approximately 10% of the complex, it was present at a concentration of 100 $\mu\text{g/ml}$. The concentration of C6 in serum is 60–80 $\mu\text{g/ml}$. A similar quantitative relationship applies to the other components in serum and in the isolated complex. The difference in intensity of the precipitin lines formed by serum and the complex indicates restricted antigenic expression of the components in the complex compared to serum.

Dissociation and Subunit Composition of the C5b-9 Complex. Various reagents were investigated for dissociation of the C5b-9 complex into subunits. Under the conditions employed, treatment with SDS was the most effective, 8 M urea was partially effective, and the other reagents tested (see Materials and Methods) were ineffective. Fig. 7 depicts the pattern of SDS-dissociated C5b-9 complex upon acrylamide gel electrophoresis in the presence of SDS. Seven major protein bands are apparent after staining with Coomassie Blue. The spectrophotometric analysis is recorded in the lower part of the figure and the pattern is representative for 22 different preparations and more than 200 individual analyses. Staining with periodic acid Schiff revealed all seven bands, with band 5 staining the most intensely.

Identification of the Subunits. To identify the subunits, five different preparations of complex were obtained from five serum samples, each containing

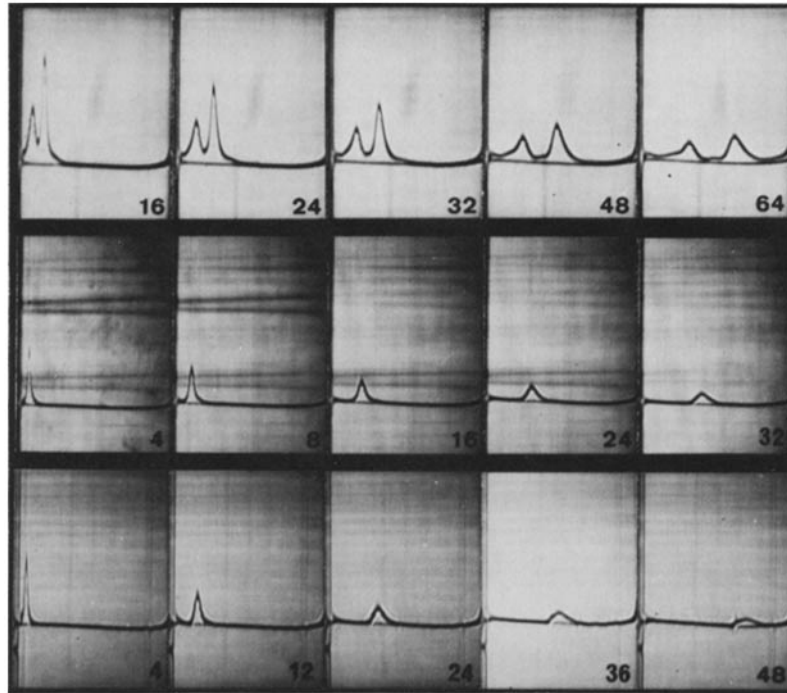


FIG. 4. Analytical ultracentrifugation of the C5b-9 complex before (upper frames) and after (middle and lower frames) removal of low density lipoprotein by flotation in CsCl. Analyses were conducted with schlieren optics employing an AN-D rotor at 52,000 rpm and 20°C. The direction of sedimentation was from left to right and the time in minutes when each picture was taken is indicated in the lower right hand corner of each frame.

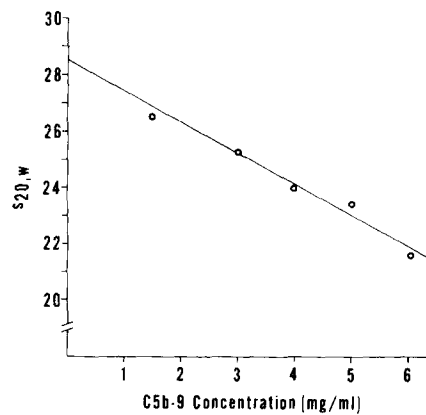


FIG. 5. Sedimentation coefficient of the C5b-9 complex as a function of concentration. Centrifugation was conducted as described for Fig. 4.

a different one of the five terminal components in radiolabeled form. Thus each complex preparation incorporated a different radiolabeled C protein. SDS gel electrophoresis of the dissociated C5b-9 preparations was combined with an analysis of the radioactivity distribution in the gels. Fig. 8 represents a composite

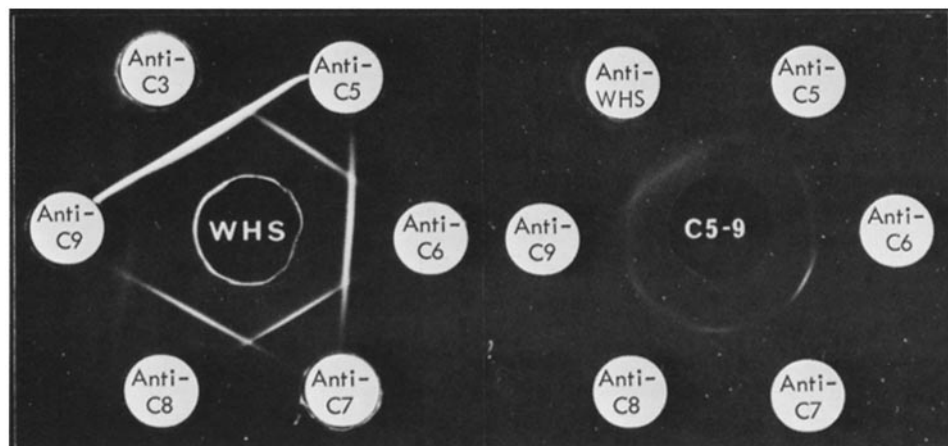


FIG. 6. Comparative immunochemical analysis of the C5b-9 complex and its precursors in WHS. Ouchterlony analysis was conducted in 1.5% agarose containing 10 mM EDTA using antisera to C5, C6, C7, C8, and C9. The center well of the right panel contained C5b-9 preparation number 8 at a concentration of 1 mg/ml. The center well of the left panel contained undiluted WHS.

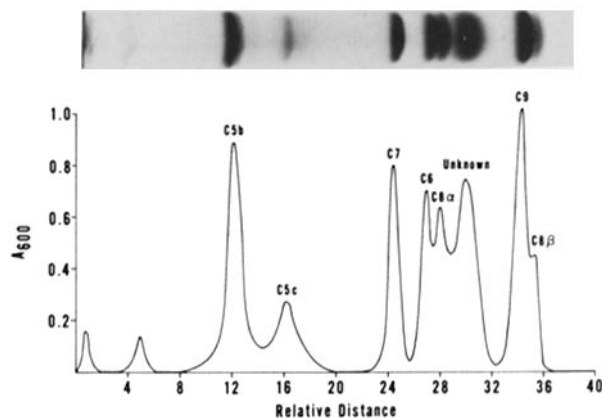


FIG. 7. Demonstration of the subunit composition of the C5b-9 complex by SDS-polyacrylamide gel electrophoresis. The isolated complex was applied after pretreatment with 0.2% SDS in the absence of any reducing agent. Electrophoresis was conducted in a 7.5% gel at 8 mA/gel and the gel was stained with 0.02% Coomassie Blue. The lower part of the figure shows the results when the gel was scanned spectrophotometrically at 600 nm. The individual components are labeled in accordance with the results demonstrated in Fig. 8.

of the results obtained, and shows that bands 1, 2, 3, and 6 could be identified, respectively, with C5, C7, C6, and C9. Bands 4 and 7 were identified as C8 α and C8 β , two noncovalently linked subunits of C8. Band 5 could not be identified with any of the known terminal C components and its nature remains presently unknown.

To verify that C5 was incorporated into the complex as C5b, the following experiment was conducted. Complex produced with [125 I]C5 was isolated, mixed

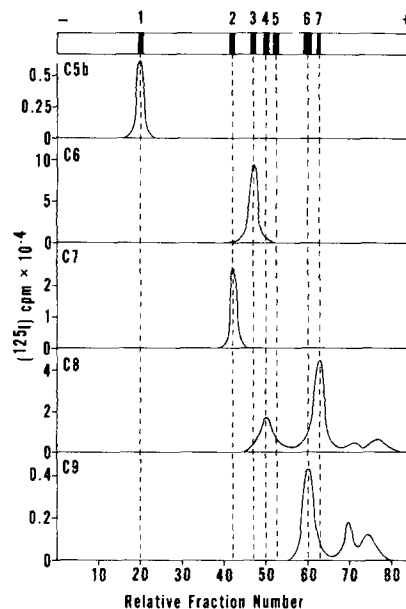


FIG. 8. Identification of the subunits of the dissociated C5b-9 complex. SDS-polyacrylamide gel electrophoresis was performed using five different SDS-dissociated complex preparations, each of which contained one of the terminal components in radiolabeled form. Electrophoresis was conducted at 8 mA/gel, the gels were stained with 0.02% Coomassie Blue, sliced into 2-mm segments, and ^{125}I radioactivity was determined.

with free ^{131}I C5, the mixture was treated with 1% SDS, and analyzed by SDS gel electrophoresis. Fig. 9 shows that C5 previously incorporated into the complex is of somewhat smaller molecular size than native C5. The mol wt difference is approximately 15,000 daltons, and therefore indicative of the presence of C5b in the complex. In addition, the complex may contain variable amounts of a C5-derived component having a mol wt of 148,000. This fragment was termed C5c (also see Fig. 7).

In addition, C8 and C9 were examined for molecular changes associated with C5b-9 complex formation by a method analogous to that used for C5. As shown in Fig. 9, incorporation of C8 or C9 was not accompanied by discernible alterations in molecular structure. The significance of the radioactivity peaks in the low molecular weight range is unknown, however, their presence is not attributable to C5b-9 complex formation.

The molecular weights of each of the seven protein bands were determined using reference protein markers. Table II lists the results and shows that the observed values are in good agreement with those published for isolated C5 to C9. The mol wt of the unidentified material (band 5) is 88,000 daltons.

Molar ratios among the subunits were estimated using the stain intensity of the bands as an approximate measure of the amount of subunit protein present (Fig. 7). As listed in Table III, C5b, C6, C7, and C8 are found in equimolar amounts, whereas C9 and the unknown component were both three times more abundant.

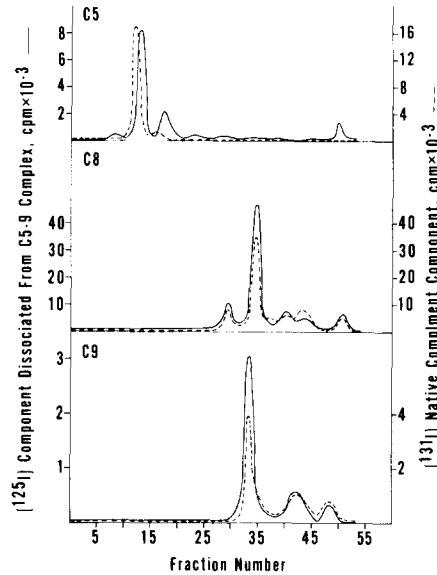


FIG. 9. Coelectrophoresis of [^{125}I]C5, -C8, or -C9 dissociated from the C5b-9 complex with native C5, C8, or C9 radiolabeled with ^{131}I . Electrophoresis was conducted at 8 mA/gel and the gels were sliced into 2-mm segments and ^{125}I and ^{131}I radioactivity were determined. For further explanation see text.

TABLE II
Composition of SDS-Dissociated C5b-9 Complex as Analyzed by
SDS-Polyacrylamide Gel Electrophoresis

Component	Published molecular weight	References	Observed molecular weight	Band no.
C5b	185,000	(12, 13)	173,000	1
C6	95,000	(4)	99,000	3
C7	120,000	(5)	110,000	2
C8	153,000	(6)	Heavy (α) 93,000 Light (β) 70,000	4 7
C9	79,000	(7)	76,000	6
Unknown	—		88,000	5

Discussion

Activation of C in whole human serum (WHS) leads to self-assembly of the terminal C proteins into a stable complex which has the transient ability to attack and to cause damage to biological membranes. In absence of target membranes, the complex accumulates in cytolytically inactive form in the fluid phase. It has previously been shown to have a mol wt of 1,040,000 daltons and the electrophoretic mobility of an α -globulin (2).

Isolation of the complex from WHS is technically simple because of its large size and pronounced negative charge. It is readily freed of IgM and α_2 -macro-

TABLE III
Molar Ratios of Proteins Dissociated from the C5b-9 Complex as Determined by Scanning of SDS Polyacrylamide Gels Stained with Coomassie Blue

Component	Stain intensity (A)*	Moles (A/Mol wt $\times 10^{-5}$)	Molar Ratio‡
C5b	3.42	1.98	0.80
C5c	1.27	0.86	0.34
C6	3.14	3.17	1.28
C7	2.74	2.49	1.00
C8 α	2.81	3.02	1.21
C8 β	1.77	2.53	1.02
C9	6.08	8.00	3.21
Unidentified	6.97	7.92	3.18

* The stain intensity (A_{600}) for each component was determined by the weight (g) of the paper corresponding to the area of the peak.

‡ Molar ratios are expressed relative to C7.

globulin. β -lipoprotein, defined immunochemically, remains, however, partially associated with the C5b-9 complex even during prolonged preparative electrophoresis. It is conceivable that the complex possesses an affinity for certain lipids or lipoproteins as an expression of its potential membrane-damaging activity. Removal of β -lipoproteins was readily achieved by ultracentrifugation in CsCl. Work is underway with highly purified complex and isolated lipoproteins to investigate their possible interactions.

The isolated complex is recognized by antibody to any of the five known precursor proteins. Compared to the reaction with the precursors, the reaction with the complex, however, is weak. Although there exists no apparent antigenic similarity between the precursors, the antisera to those five components react with the complex in gel diffusion tests producing a single fusing precipitin line without spur formation. The observations indicate that the complex does not dissociate during the immunochemical analysis, that some antibody-reactive groups of C5, C6, C7, C8, and C9 are exposed on its surface, and that a major portion of their antigenic expression is concealed interiorly. These observations are consistent with the previously proposed compact model of the fully assembled complex (1).

The complex is of remarkable stability, although there is no evidence for covalent bonding between the self-assembled subunits. They are completely dissociated by SDS in the absence of any reducing agents. Dissociation allowed analysis of subunit composition and the establishment of the following facts: (a) C5 is contained in the complex as C5b, as evidenced by molecular weight reduction. (b) The size of the other C proteins in the complex is indistinguishable from that of the respective precursors. (c) Precursor and complex C8 consist of two noncovalently bound subunits of different size. (d) C9 is incorporated with a molar multiplicity compared to C5, C6, C7, and C8. (e) An unknown subunit was

invariably found which is distinct from known C proteins, has a mol wt in SDS of 88,000 daltons and appears to contain a considerable amount of carbohydrate as revealed by periodic acid Schiff staining of polyacrylamide gels.

The fact that C5b is found in the complex and not C5, whereas all other proteins are apparently chemically unchanged by the incorporation, is inconsistent with the concept proposing enzymatic cleavage of C5 as the biochemical signal for an adsorptive fusion of C5b-9 (2). The presence in the complex of a C5-derived minor component (C5c) with a molecular weight lower than that of C5b may be due to secondary cleavage of bound C5b by C3b inactivator or conglutinin-activating factor (KAF) (14, 15). KAF not only cleaves C3b (16) but apparently also C4b.³

The most immediate question to be answered regards the nature and function of the unidentified band 5 material. It is contained in the complex in an equimolar amount compared to C9. Therefore the complex contains three molecules of the 88,000 dalton species or one molecule of 264,000 daltons consisting of three noncovalently linked subunits of equal molecular weight. Work is underway to define the protein by immunochemical techniques. As to its function, three possibilities are considered, namely that it represents a serum equivalent of a membrane receptor for nascent C5b-9, an inhibitor of the complex, or a new C protein.

The most challenging question resulting from this work concerns the manner in which the C5b-9 complex impairs biological membranes. The unique activity generated by this set of soluble proteins has in all probability its basis in a unique structural characteristic. More work is needed to determine whether the complex or any of its subunits exhibit an unusual structural property.

Summary

Isolation of the C5b-9 complex from inulin-activated whole human serum was effected by molecular sieve column chromatography employing Biogel A-15 M, preparative Pevikon block electrophoresis, and removal of low density β -lipoproteins by flotation in CsCl. The final product was homogeneous upon cellulose acetate strip electrophoresis and analytical ultracentrifugation. Ouchterlony analyses indicated that the complex reacted with antisera to C5, C6, C7, C8, and C9 to form a continuous, circular precipitin line without spurs.

The C5b-9 complex was dissociated by sodium dodecyl sulfate (SDS) in the absence of reducing agents, and analytical SDS-polyacrylamide gel electrophoresis revealed seven protein bands after staining with Coomassie Blue. Bands 1, 2, 3, and 6 were identified as C5b, C7, C6, and C9, respectively. Bands 4 and 7 were identified as two noncovalently bound subunits of C8. Molar ratios among C5b, C6, C7, C8, and C9 dissociated from the complex by SDS were estimated to be 1:1:1:1:3. Band 5 protein, which had an estimated mol wt of 88,000 and was found to occur with a molar ratio of 3, has not yet been identified. Its nature and possible biological functions are discussed.

The authors wish to thank Ms. Judy Meinkoth for her excellent technical assistance.

³ Cooper, N. R. Manuscript in preparation.

Received for publication 9 December 1974.

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