MOLECULAR ORGANIZATION OF C9 WITHIN THE MEMBRANE ATTACK COMPLEX OF COMPLEMENT

Induction of Circular C9 Polymerization by the C5b-8 Assembly*

By ECKHARD R. PODACK,‡ JÜRG TSCHOOP,§ AND HANS J. MÜLLER-EBERHARD‖

From the Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

One of the characteristics of the membrane attack complex (MAC)[1] is that it contains multiple C9 molecules in comparison with its other molecular constituents C5b, C6, C7, and C8 (1–4). Recently, isolated C9 was found to polymerize spontaneously and form tubular structures in a time- and temperature-dependent reaction (5). Polymerized C9 (poly C9) consists of 12–16 C9 molecules and has a molecular weight of ~1,100,000 (6). The tubules are 16 nm long and have an inner and outer diameter of 10 and 21 nm, respectively. The ultrastructural appearance of poly C9 resembles closely that of the isolated MAC (5, 7, 8). During polymerization, C9 acquires the ability to become inserted into phospholipid bilayers, render lipid vesicles leaky, fuse such vesicles, or disassemble their membrane.[2] Despite these properties, poly C9 by itself could not be shown to be cytolytic for nucleated cells or erythrocytes from a variety of species.

Because of the C9 multiplicity within the MAC and the ultrastructural and functional properties of poly C9, the possibility arose that poly C9 is a regular constituent of the fully assembled MAC, and, in fact, constitutes its putative hydrophilic transmembrane channel (9, 10). This paper indicates that MAC assembly includes the process of poly C9 formation and that the typical ultrastructural membrane lesions caused by complement are the images of poly C9 formed by membrane-bound C5b-8. Part of this work has been presented in an abstract (11).

Materials and Methods

Complement Proteins. C5b-6, C6, C7, and C8 were purified as described elsewhere (12–14). C5 and C9 were purified by DEAE-Sephacel chromatography according to the method of

* Supported by grants AI-17354, AI-18525, CA-27489, and HL-16411 from the U. S. Public Health Service. Publication 2681 from the Research Institute of Scripps Clinic, La Jolla, CA.

‡ Recipient of Established Investigatorship 79-149 from the American Heart Association.

§ Recipient of a postdoctoral fellowship from the Swiss National Science Foundation.

‖ Cecil and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic, La Jolla, CA.

[1] *Abbreviations used in this paper:* DOC, desoxycholate; MAC, membrane attack complex of complement; poly C9, polymerized C9 consisting of 12–16 C9 protomers; SDS, sodium dodecyl sulfate.

[2] Tschopp, J., H. J. Müller-Eberhard, and E. R. Podack. Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9. Manuscript submitted for publication.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/07/0268/15 $1.00
Volume 156 July 1982 268–282

Hammer et al. (15). The final step for C5 purification was chromatography on concanavalin A-Sepharose CL4B to remove traces of contaminating IgG. The post DEAE-Sephacel C9 pool was further purified to homogeneity by hydroxyapatite chromatography as described (16). The C8 and C9 concentration was determined by the absorbance at 280 nm using extinction coefficients of $\epsilon = 1.6$ and $\epsilon = 0.96$ ml mg^{-1} cm^{-1}, respectively (5). Poly C9 was prepared by incubating purified C9 at 1 mg/ml for 64 h at 37°C in Tris- (10 mM) buffered saline (0.15 M), pH 7.4, containing 25 μg soybean trypsin inhibitor. Poly C9 was devoid of monomeric C9 (4.5S) as determined in the analytical ultracentrifuge.

Radiolabeling of C5, C7, C8, and C9 was carried out with the iodogen procedure (17) to reach specific radioactivities of 5×10^5 cpm/μg protein. Functional activities of labeled proteins were tested by their incorporation into the SC5b-9 complex as determined by sucrose density gradient ultracentrifugation. C9 was tested as follows: 0.1 ml of whole human serum containing 10 mM EDTA was supplemented with 10^5 cpm ^{125}I-C9 and with 10 μg C7 and 15 μg C8. SC5b-9 formation was then initiated by the addition of 30 μg C5b-6 and incubation for 30 min at 37°. Controls were incubated without C5b-6. Samples were then applied to 10–40% linear sucrose density gradients dissolved in veronal-buffered saline, pH 7.4, containing 10 mM EDTA, and centrifuged for 16 h at 36,000 rpm at 4°C in a SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, CA.). After fractionation, the amount of ^{125}I-C9 sedimenting with 23S (18) was determined. The other proteins were examined similarly. The binding activity of the labeled proteins ranged from 50 to 80%.

The binding activity of unlabeled proteins was determined by two-dimensional immunoelectrophoresis as described here for C9: immunochemically C9-depleted serum was reconstituted with 50 μg/ml purified C9, and purified C7 and C8 were added to a final concentration of 100 μg/ml. C5b-6 was added at concentrations ranging from 0 to 250 μg/ml and the mixture was incubated for 30 min at 37°C. 7-μl aliquots were then subjected to two-dimensional immunoelectrophoresis using anti-C9 in the second dimension. The disappearance of the C9 peak on addition of increasing amounts of C5b-6 indicated the formation of the SC5b-9 complex, which has a lower electrophoretic mobility and did not react with the antiserum under these conditions. All of the C9 antigen was bound to SC5b-9 when C5b-6 was at one-half the molarity of the C9. Thus, 100% of the purified C9 antigen and 100% of C8 antigen, measured similarly, was active. Comparison of protein determinations with determinations of antigen by the Mancini technique indicated that essentially all the protein represented immunochemically reactive C8 and C9.

Determination of C9/C8 Molar Ratio and Preparation of Membranes for Electron Microscopy. The C8- and C9-binding assays are based on the virtually irreversible binding of C8 and C9 to membranes bearing C5b-7 (4). When constant trace amounts of radiolabeled C8 and C9 and various amounts of unlabeled C8 and C9 participate in MAC assembly on erythrocyte membranes, competition for binding sites between labeled and unlabeled components occurs only when the total C8 or C9 exceeds all available binding sites. Thus, by determining the minimum amount of unlabeled protein that detectably decreases uptake or radiolabel, the number of bindings sites can be calculated (Fig. 1). This assay is not influenced by possible inactivation of proteins during the radiolabeling procedure.

Normal human serum was depleted of C8 and C9 by immune adsorption with the aid of antibodies coupled to Sepharose CL4B (13). Depletion was assessed by two-dimensional immunoelectrophoresis and hemolytic titration of C8 and C9. 15,000 cpm ^{131}I-C8 was added to 150-μl serum aliquots for C8 uptake studies. Each aliquot then received various amounts (0–4 μg) of unlabeled C8 (see Fig. 1) and 50 μl of rabbit erythrocytes adjusted to a 25% hematocrit ($\sim 2.5 \times 10^9$ cells/ml). The mixtures were incubated for 1 h at 37°C in a total vol of 250 μl. 125 μl of each incubation mixture was applied to a microfuge tube containing 250 μl of 10% sucrose dissolved in veronal-buffered saline, pH 7.4, containing 0.5 mg/ml ovalbumin, 10 mM benzamidine hydrochloride, and centrifuged for 5 min (model B microfuge; Beckman Instruments, Inc.). Under these conditions, both unlysed cells and cell membranes were pelleted. The tip of the microfuge tube containing the pellet was cut off with a razor blade and the radioactivity in pellet and supernate was measured. The remainder of the incubation mixture not used for determination of C8 uptake was used for electron microscopy. After addition of 0.5 ml 0.5 mM phosphate buffer, pH 8.0, the membranes were sedimented in the microfuge,

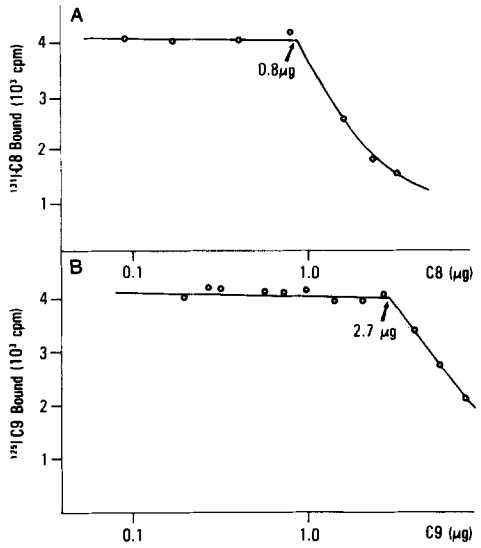


FIG. 1. Molar ratio of C8 and C9 bound to rabbit erythrocytes as a result of MAC-assembly. To obtain the C8-binding curve, 1.25×10^8 rabbit erythrocytes were incubated with 150 μl of C8- and C9-depleted human serum reconstituted with a trace of ^{131}I-C8 and various amounts of unlabeled C8. After 1 h at 37°C, specific uptake of radiolabeled C8 was measured and plotted as a function of input of total C8 (A). Controls were incubated in the presence of EDTA and showed <1% unspecific C8 binding. The inflection point of the binding curve corresponds to the number of C8-binding sites (0.8 μg = 3.2×10^{12} molecules). C9 binding was determined in an analogous fashion (lower panel). The weight ratio of 2.7 μg C9 per μg C8 corresponds to a molar C9/C8 ratio of 6.7.

washed twice with the phosphate buffer, and resuspended in 200 μl veronal- (3.3 mM) buffered saline (0.15 M), pH 7.4. Trypsin and chymotrypsin were added to a final concentration of 100 μg/ml and the same incubated overnight at room temperature. After two washes with veronal-buffered saline and resuspension in 50 μl, the samples were mounted on electron microscopy grids, stained with 2% uranyl formate, and examined in a Hitachi 12A (Hitachi Tokyo Ltd.), electron microscope.

C9 uptake was measured similarly by the addition of 15,000 cpm ^{125}I-C9 to serum aliquots reconstituted with defined amounts of unlabeled C8 and ^{131}I-C8. Unlabeled C9 (0–10 μg) was then added and the aliquots incubated with rabbit erythrocytes as described above. Uptake of ^{125}I-C9 in dependence of the amount of unlabeled C9 was determined as outlined above for C8. In experiments measuring C9 binding at 4°C, ^{131}I-C5b-8 was first formed on rabbit erythrocytes at 37°C for 1 h. ^{125}I-C9 and unlabeled C9 were then added to chilled aliquots and parallel samples incubated at 37°C and on ice.

C9-binding Sites on C8 in Solution. C9-binding sites on C8 were determined in the analytical ultracentrifuge by mixing purified C8 and C9 at various molar ratios in 50 mM NaCl, 10 mM Tris HCl, pH 7.4, and centrifugation at 52,640 rev/min in an ANF 60 rotor (Beckman Instruments, Inc.). The protein concentrations of C8 and C9 used are listed in Table I. The molar ratio of the 10.5S C8-C9 complex formed was determined from its absorbance of light at 280 nm and from the amount of remaining free C9 (4.5S).

Analysis of MAC-associated Poly C9 by Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gradient Slab Gel Electrophoresis. MAC-containing ^{125}I-C9 and ^{131}I-C7 was formed on and extracted from rabbit erythrocytes as described (8). The desoxycholate (DOC) extract was further purified by chromatography over Sepharose CL4B in the presence of DOC (8). For SDS gel electrophoresis, MAC-containing fractions were made 10% (wt/vol) in SDS and, under reducing conditions, 2% in mercaptoethanol. After boiling for 2–5 min, samples were applied to SDS-polyacrylamide gradient slab gels and electrophoresis conducted using the discontinuous buffer system described by Laemmli (19). The 6-cm-long gradient ranged from 2.5–10% polyacrylamide overlayered by

TABLE I

Reversible Interaction of C8 and C9 in Solution Analyzed by Analytical Ultracentrifugation

Input			Sedimentation coefficients	C8 free*	C9 free*	C8:C9 in 10.5S complex‡
C8	C9	C8:C9				
μg/ml	*μg/ml*	*mol/mol*	*S*	*μg/ml*	*μg/ml*	*mol/mol*
0	150	0:1	4.5	NA§	150	NA
300	0	1:0	8.1	300	NA	NA
300	150	1:1	10.5	0	0	1
300	225	1:1.5	10.5, 4.5	0	75	1
300	300	1:2	10.5, 4.5	0	150	1
300	450	1:3	10.5, 4.5	0	300	1

* Protein concentration determined from the absorbance of light at 280 mm of the 8.1S or 4.5S boundary, respectively.

‡ Molar ratio determined from the observed sedimentation rate of 10.5 r_S corresponding to a 1:1 C8:C9 complex and from the absorbance of light at 280 nm.

§ Not applicable.

2 cm 2.5% polyacrylamide and the stacking gel that was also formed by 2.5% polyacrylamide. The samples were subjected to electrophoresis for 16 h at 25 V, constant voltage, stained with Coomassie blue and, where indicated, sliced into 2-mm segments for determination of radioactivity. Densitometric scanning of stained bands was carried out in a soft laser scanning densitometer (Biomed Instruments, Inc., Chicago, IL).

Immunochemical Analysis. Antisera to poly C9 were raised in rabbits by eight injections of poly C9. Double-diffusion analysis was performed in 1% agarose containing 1% Triton-X 100.

Results

Formation of a Reversible Equimolar C8-C9 Complex in Solution. The number of C9-binding sites on a molecule of C8 was determined by analytical ultracentrifugation. The results are listed in Table I. Whereas C8 has a sedimentation coefficient of 8.1S (14) and C9 of 4.5S (20), an equimolar mixture of C8 and C9 sedimented as a single 10.5S component, indicating the formation of a stoichiometric C8-C9 complex. When the molar concentration of C9 was raised above that of C8, a 4.5S component was observed in addition to the 10.5S component. The results indicate that C8 possesses a single binding site of C9.

Binding of Multiple C9 Molecules to Membrane-bound C5b-8. To determine the molar ratio of C8 and C9 bound to membranes upon MAC formation, a sensitive assay was used that takes advantage of the high affinity of C9 ($K_a \sim 10^{11}\ M^{-1}$) (4) for membrane-bound C5b-8, as described in Materials and Methods. Briefly, normal human serum was immunochemically depleted of C8 and C9 and then reconstituted with trace amounts of ^{131}I-C8 and ^{125}I-C9 and with various amounts of unlabeled C8 and C9. After incubation of serum samples with rabbit erythrocytes for 1 h at 37°C, the amount of membrane-bound radioactivity was determined and related to input of unlabeled C8 or C9 (Fig. 1). The maximum amount of unlabeled protein that did not cause a reduction of binding of radioactivity to the cells was considered equivalent to the number of available binding sites for that protein. In the case of C8, this amount was 0.8 μg (Fig. 1 A) and for C9 it was 2.7 μg (lower panel).[3] These values correspond

[3] It is important that C8 and C9 are present simultaneously during MAC assembly on rabbit erythrocyte membranes. If C5b-8 is first formed for 1 h at 37°C and C9 uptake measured in a subsequent incubation for 1 h at 37°C, the molar C9/C8 ratio drops to 3.8 and the membrane lesions have an irregular, fused appearance. Presumably, C5b-8 complexes cluster and thereby restrict subsequent C9 binding.

TABLE II

Dependence of the Molar C9/C8 Ratio of EC5b-9 on the Number of C8 Molecules Per Cell

C5b-7 complexes/cell	C8 molecules/cell	C9 molecules/cell	C9:C8 ratio*	Number of determinations
2.5×10^4	2.5×10^4	16.6×10^4	6.6 (5.2–7.8)	6
2.5×10^4	6.4×10^3	5.6×10^4	8.8 (7.3–11.4)	3
2.5×10^4	3.2×10^3	3.7×10^4	11.6 (10.4–12.4)	3
2.5×10^3	2.5×10^3	4.4×10^4	15.4 (12.2–21.8)	4

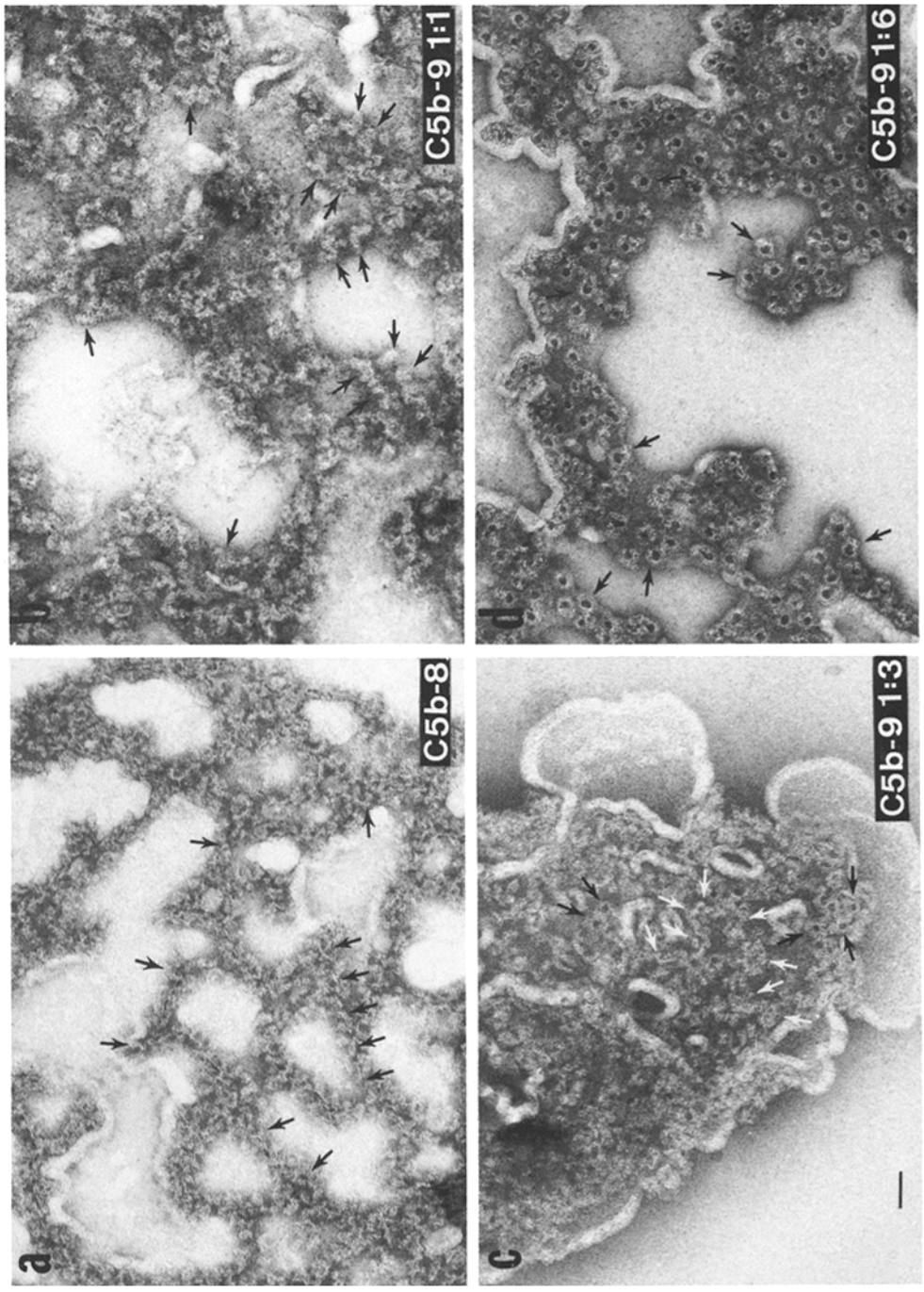
* Mean value; observed range shown in parentheses.

to 2.56×10^4 C8 molecules and 1.72×10^5 C9 molecules per cell or 6.75 molecules C9 per C8 molecule. This complex was assigned the formula $(C5b\text{-}8)_2$polyC9 consistent with the dimer hypothesis of MAC structure. At 0°C, the binding of C9 to EC5b-8 was reversible and the association constant derived from the midpoint of the binding curve was determined to be $\sim 3 \times 10^7\ M^{-1}$ (data not shown).

Considering the possibility that singly spaced C5b-8 complexes—as opposed to clusters of such complexes—on the surface of a cell may differ in their C9-binding capacity, the following experiment was performed. Rabbit erythrocytes containing 25,000 C5b-7 and 3,200–25,000 C8 molecules per cell were exposed to an excess of C9, and C9 uptake was then measured. The results are recorded in Table II. At 25,000 C8 molecules per cell, an average of 6.6 C9 molecules were bound per C8 molecule. At 3,200 C8 molecules per cell, an average of 11.6 C9 molecules were bound per C8 molecule. When 2,500 C8 molecules were bound to an equal number of C5b-7 molecules, C9 binding averaged 15.4 molecules per C8 molecule. These results suggest that separately spaced single C5b-8 can mediate poly C9 formation. Such complexes were assigned the structural formula (C5b-8)polyC9.

Dependence of the Ultrastructural Appearance of Membrane-bound C5b-9 on the C9 Content of the Complex. Membranes of rabbit erythrocytes bearing 25,000 C5b-8 complexes and various numbers of C9 molecules were examined by electron microscopy (Fig. 2). Membranes bearing C5b-8 but no C9 exhibited a large number of half-ring-like structures (Fig. 2 A). These structures appeared to become aggregated on membranes with C5b-9 complexes that contained one molecule of C9, suggesting that C5b-8 complexes may have become cross-linked by C9-C9 interaction at low C9 dose (Fig. 2 B). At a molar ratio of three C9 per C8, a small number of ring structures are visible (Fig. 2 C). When six C9 molecules were bound per C8 molecule, the characteristic complement-dependent membrane lesion or MAC image became apparent (Fig. 2 D).

FIG. 2. Ultrastructural comparison of membrane bound C5b-8 and C5b-9 of varying C9/C8 molar ratios. Erythrocyte membrane-C5b-8 or C5b-9 complexes of defined C9/C8 molar ratios (as determined in Fig. 1) were washed with 5 mM phosphate buffer, pH 8.0, and treated with trypsin and chymotrypsin (100 μg/ml) overnight at room temperature. After further washing, the membranes were mounted on grids, stained with 2% uranyl formate, and imaged in the electron microscope. (A) Membrane-bound C5b-8 imaged as clustered half-ring-like structures (arrows). The erythrocyte membranes are highly disrupted (white areas devoid of negative stain). (B) C5b-9 at 1:1 molar C8/C9 ratio appears as large, condensed aggregates (arrows), suggesting cross-linking of C5b-8 complexes by C9 at low C9 multiplicity. No circular structures are apparent. (C) C5b-9 at 1:3 molar C8/C9 ratio with occasional ring structures (black arrows) and the majority of complexes forming continuous arrays of C5b-9 aggregates (white arrows). (D) Typical round lesions are seen at a 1:6 molar C8/C9 ratio. Bar, 400 Å.


a
C5b-8
b
C5b-9 1:1
c
C5b-9 1:3
d
C5b-9 1:6

These structures, which have the probable composition (C5b-8)$_2$polyC9, are clearly delineated and largely unaggregated.

As shown above, individual singly spaced C5b-8 could mediate the binding of 12–15 C9 molecules. These cells, bearing 25,000 C5b-7, 3,200 C8, and 38,000 C9 molecules, displayed individual ring-like structures that were widely separated from each other on their surface (Fig. 3 B). The molecular composition of these structures, presumably, is (C5b-8)poly C9. For comparison, cells are shown that bear the same number of C5b-7 complexes with, however, 25,000 C8 molecules and 167,000 C9 molecules (Fig. 3 A). The inset (Fig. 3) shows isolated poly C9 for comparison. In top views, poly C9 has a circular structure that is identical to that of the MAC.

Demonstration of Poly C9 as a Constituent of the MAC. These results indicate that

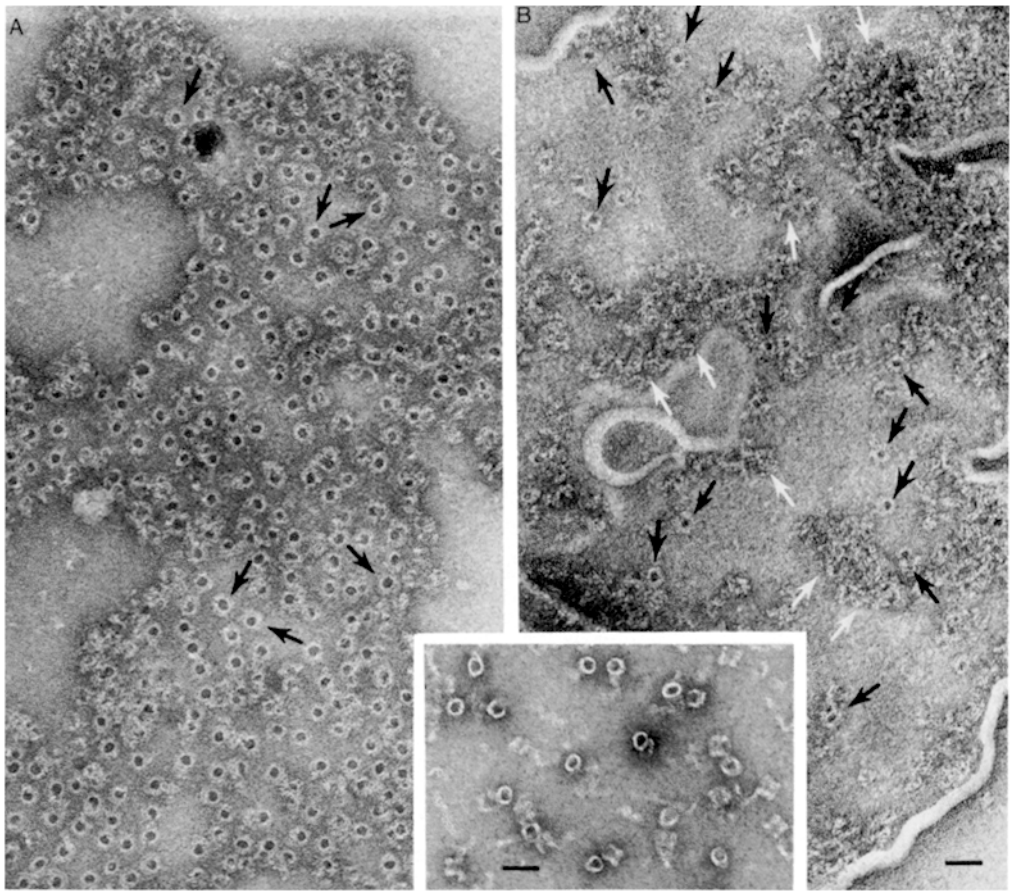


FIG. 3. Comparative ultrastructural analysis of membranes containing complexes with the composition (C5b-8)$_2$poly C9 (A) and (C5b-8)polyC9 (B). (C5b-8)$_2$polyC9 formed when 25,000 C5b-7 complexes per cell bound 25,000 C8 molecules and 167,000 C9 molecules. The resulting complexes are imaged as typical complement lesions, many of which exhibit a fine structure of poly C9. (C5b-8)polyC9 formed when 25,000 C5b-7 complexes per cell bound 3,200 C8 and 38,000 C9 molecules. Widely spaced typical lesions (B) are visible (black arrows) in addition to aggregated C5b-7 complexes (white arrows), which did not bind C8 or C9. (Inset) the ultrastructural image (top and side views) of isolated poly C9 are shown for comparison. Bar, 400 Å.

whereas one molecule of C8 in solution can bind only one molecule of C9, a membrane-bound C8 molecule, in the form of the C5b-8 complex, can mediate the binding of many C9 molecules. It was, therefore, examined whether C5b-8 can induce C9 polymerization. Direct evidence for the occurrence of poly C9 in the MAC was obtained by subjecting the isolated complex containing C9 in radiolabeled form to SDS-polyacrylamide gradient slab gel electrophoresis. The MAC was isolated from rabbit erythrocytes after treatment with human serum containing ^{125}I-C9 and ^{131}I-C7. Fig. 4 shows the distribution of protein and of C9 radioactivity throughout the gel. In addition to monomeric C9 (M_r = 71,000) and dimeric C9 (M_r = 155,000), a large proportion (43.9%) of high molecular weight C9 is seen. The position of this material in the 2.5–10% gel indicates a molecular weight of ~1,100,000, suggesting that the polymer contains 12–16 C9 monomers. Not only is this polymer resistant to dissociation by SDS in nonreduced form, but it is also resistant to dissociation under reducing conditions as shown in Fig. 5. The properties of the MAC-associated high molecular weight C9 are, with respect to molecular weight and resistance to SDS and reducing agents, characteristic of the recently described poly C9 (5), the tubular structure that is capable of forming channels through lipid bilayers. Fig. 6 shows an SDS slab gel electrophoresis analysis of isolated C9 after incubation for 64 h at 37°C. During incubation, C9 underwent spontaneous polymerization, and ~50% of poly C9 became resistant to dissociation by SDS and reducing agents and exhibited an apparent molecular weight of 1.1 million.

An antiserum produced to poly C9 detects, on the polymer, antigenic determinants that are not present on monomeric C9. An Ouchterlony analysis using anti-poly C9 shows the precipitin line of poly C9 heavily spurring over that of monomeric C9 (Fig. 7, center). The same precipitin pattern is observed when the isolated MAC is

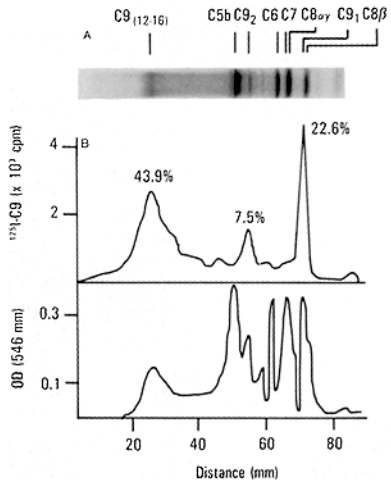


FIG. 4. Demonstration of SDS-resistant poly C9 as constituent of the MAC by SDS-polyacrylamide gradient slab gel electrophoresis. The 8-cm-long gel contained 2.5–10% polyacrylamide and was overlayered by a 2.5% stacking gel. 50 μg isolated MAC was applied to the slab in SDS sample buffer under nonreducing conditions and subjected to electrophoresis for 16 h at 25 V. The Coomassie blue-stained gel is shown together with the distribution of C9-associated radiolabel (A) and the densitometric scan of the dye distribution in the gel (B). The mol wt of poly C9 was estimated to be 1.1 × 10^6 by comparison with marker proteins.

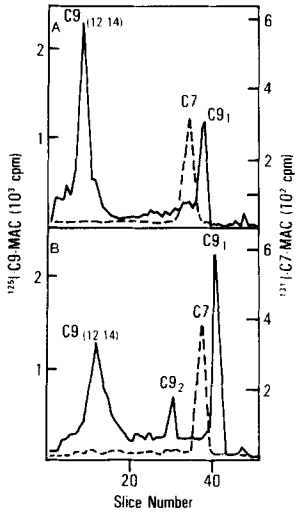


FIG. 5. Resistance of MAC-associated poly C9 to dissociation by SDS under reducing conditions. The MAC containing ^{131}I-C7 and ^{125}I-C9 was isolated as described and subjected to SDS-polyacrylamide gradient gel electrophoresis as described in Fig. 4. The gel was sliced into 2-mm sections for radioactivity determinations. After reduction (A) poly C9, monomeric C9, and C7 migrate slightly slower than the nonreduced proteins. The C9 dimer (mol wt ≃ 155,000) was converted to the monomer on reduction.

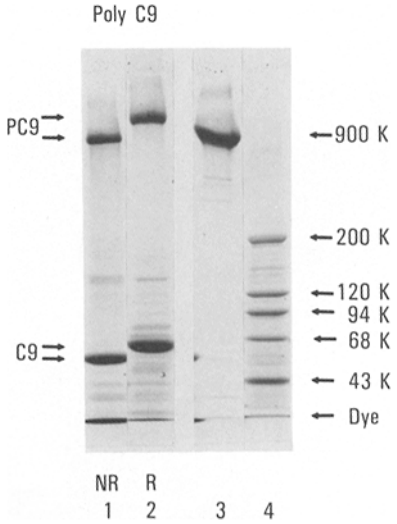


FIG. 6. Resistance of isolated poly C9 to dissociation by SDS under reducing (R) and nonreducing (NR) conditions. C9 was polymerized at 1 mg/ml in Tris-buffered saline at 37° C for 64 h. 20 μg was applied to the polyacrylamide gradient slab (2.5–10%) without (track 1) or with (track 2) prior reduction. Approximately 50% of the polymerized C9 was resistant to dissociation to SDS. Track 3 represents IgM (mol wt ≃ 900,000) and track 4 marker proteins with the indicated molecular weights K, 1,000 mol wt.

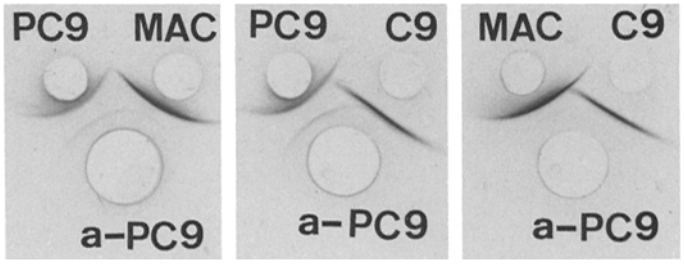


FIG. 7. C9-derived neoantigens in the MAC and in poly C9. The wells contained 12 μg MAC, 7 μg C9 or poly C9, and 15 μl antiserum prepared by immunizing rabbits with poly C9. 1% agarose containing 1% Triton-X 100 was used for double diffusion at 4°C to prevent polymerization of monomeric C9.

compared with monomeric C9 (Fig. 7, right). That the same neoantigens occur in the MAC and in poly C9 is shown by the precipitin pattern of apparent identity (Fig. 7, left).

Discussion

The results indicate that poly C9 may be a regular constituent of the MAC and that C9 polymerization is induced by membrane-bound C5b-8. The evidence for the occurrence of poly C9 in the MAC is the following: (*a*) poly C9 ($M_r \simeq 1{,}100{,}000$) was clearly detectable by SDS-gel electrophoresis of MAC isolated from complement-lysed erythrocytes. Like poly C9 produced by prolonged incubation at 37°C from isolated C9 (5), the MAC-derived poly C9 was resistant to dissociation by SDS and reducing agents; (*b*) the MAC contains many C9 molecules; (*c*) identical neoantigens were expressed by poly C9 and the MAC; and (*d*) the ultrastructural image of the MAC resembles that of poly C9 (5). Because isolated poly C9 inserts itself into lipid bilayers and renders liposomes leaky (21), it is probably that poly C9 provides the transmembrane protein channel that has been postulated to be an integral structure of the MAC (9).

Instead of all five MAC precursor proteins, as originally envisaged, only C9 forms the channel wall. The manner in which polymerization proceeds is largely unknown. The spontaneous polymerization of C9 requires a much greater activation energy than that mediated by C5b-8 (5). A molecule of C8 in solution has one C9 binding site and C8-C9 association is reversible ($K_a \simeq 10^7\ M^{-1}$). However, a cell-bound C8 molecule (C5b-8) can mediate the binding of many of C9 molecules and that association is virtually irreversible ($K_a \simeq 10^{11}\ M^{-1}$) (4). It may be assumed, therefore, that the C9-binding site on C8 within the C5b-8 organization becomes the C9 polymerization site. We postulate that binding of a molecule of C9 to C5b-8 facilitates C9-C9 interaction and that the energy derived from that interaction effects the unfolding of monomeric C9 (long axis: $\simeq$ 80 Å) to the poly C9 subunit (long axis: $\simeq$ 160 Å), which is amphiphilic and capable of inserting itself into hydrophobic environments. No enzymatic function has been attributed to C8 or its subunits. However, C6 has been reported to contain a catalytic site that hydrolyzes acetyl glycyl-lysine methyl ester and which can be inactivated by binding of diisopropyl-phosphorofluoridate (22). There is some indication that C7 has similar properties (23). End group analysis of C9 and poly C9 is needed to ascertain whether or not proteolytic attack is involved in the polymerization reaction.

C5b-9 may vary greatly in its composition and ultrastructural appearance, depending on C9 supply and the cell surface distribution of its precursor, C5b-8. When individual C5b-8 complexes are exposed to an excess of C9, they bind 12–16 C9 molecules, and by electron microscope analysis of the cell surface, individual, widely separated poly C9 rings are visualized. Such complexes were assigned the composition (C5b-8)polyC9. When clusters of C5b-8 were saturated with C9, an average of 6–8 C9 molecules were bound per complex. A large number of typical poly C9 rings was seen on those cells in close proximity. These complexes were assigned the composition $(C5b\text{-}8)_2$polyC9. We suggest that in the former situation, one poly C9 structure is generated by one polymerization site (C5b-8), whereas in the latter situation, one poly C9 is formed by two such sites or by a C5b-8 dimer.

If the supply of C9 is limited and the density of membrane-bound C5b-8 is high, poly C9 rings may not form at all. Instead, multiple C5b-9 complexes may aggregate within the membrane due to C9-C9 interaction. Heavy aggregation was observed at molar C9/C8 ratios of 1:1 and 3:1 (Fig. 2). Although discrete protein channels may not be formed under these conditions, the formation of a network of C5b-9 aggregates may be an efficient mechanism of labilizing a biological membrane. However, when cells are lysed by serum in vitro and complement activation via the classical or alternative pathway is pronounced, the prevalent MAC species generated has the composition $(C5b\text{-}8)_2$polyC9. Because the concentration of the MAC precursor proteins in serum is relatively high, C5b-9 will be deposited on the target cell at a high density and, therefore, conditions characteristic of a molar C9/C8 ratio of 6–8 pertain.

From the foregoing analysis, it is evident that the molecular weight of the MAC may vary widely depending on its subunit composition. The calculated molecular weight of (C5b-8)polyC9 is $\sim 1.6 \times 10^6$, that of $(C5b\text{-}8)_2$polyC9 is $\sim 2.2 \times 10^6$, and that of multimers of the MAC, several million. In fact, published values range from one million (24) to several million (8, 25).

The various forms of C8-C9 interaction that occur as part of the MAC assembly are summarized in Table III. Reaction 1 represents the reversible association of one molecule of C8 and one molecule of C9 in solution; 2, the reversible association of C9 with C5b-8 on cells at 0°C; 3, the irreversible binding of C9 to C5b-8 at 37°C with concomitant C9 activation (C9*); 4, the aggregation of C5b-9 at low C9/C8 molar ratio; 5, the formation of poly C9 by an individual C5b-8 in C9 excess; and 6, formation of poly C9 by two clustered C5b-8 (dimer).

Previous estimates of the number of C9 molecules in the C5b-9 complex have

TABLE III

Various C8-C9 Interactions Occurring During MAC Formation

(1)	$C8 + C9 \rightleftharpoons C8,C9$
(2)	$C5b\text{-}8 + C9 \overset{0°C}{\rightleftharpoons} C5b\text{-}8,C9$
(3)	$C5b\text{-}8 + C9 \xrightarrow{37°C} C5b\text{-}8,C9^*‡$
(4)	$C5b\text{-}8,C9^*‡ \xrightarrow{37°C} (C5b\text{-}8,C9)_n$
(5)	$C5b\text{-}8,C9^*‡ + C9 \xrightarrow{37°C} (C5b\text{-}8)$polyC9§
(6)	$C5b\text{-}8,C9^*‡ + C9 \xrightarrow{37°C} (C5b\text{-}8)_2$polyC9§

‡ C9*, activated C9 capable of inducing C9-C9 interaction.
§ Poly C9, consisting of 12–16 C9 molecules.

varied between one and six (1–4, 26). The original quantitation of maximum C9 binding capacity yielded a value of six per C8 molecule (2). The number of C9 molecules needed to produce one functional lesion was estimated to be 1–2 (26) and 3–6 (2). When it became possible to examine the composition of the isolated MAC directly by SDS-polyacrylamide gel electrophoresis, two to three molecules of C9 were found per molecule of C5b, C6, C7, or C8 (3, 8, 18). Poly C9 escaped detection by this technique and was found only when SDS-polyacrylamide gradient slab gel electrophoresis was used as reported in this paper. The SDS-resistant polymers (Fig. 4) probably constitute complete ring structures, and the monomeric C9 that was also present may have been derived from incompletely polymerized, SDS-susceptible oligomers. The nature of the forces that are responsible for the strong intermolecular interactions within poly C9 remains to be identified.

It should be borne in mind that C5b-8 even without C9 displays a certain degree of membranolytic activity. The complex can lyse erythrocytes (20, 27) and kill serum-sensitive *Neisseria gonorrhoeae* (28), and individuals with homozygous C9 deficiency do not seem to have an increased susceptibility to bacterial infections, whereas individuals with other complement deficiencies do (29–33). Apparently, the phospholipid-binding capacity of C5b-8 (34, 35) and the ability of the complex to reorganize and thereby weaken membrane structure (36) are sufficient to impair the normal function of some biological membranes. On the other hand, C9 appears essential for the killing of *Escherichia coli* (37), Raji cells (38, 39), and, presumably, many other nucleated cells by complement. The role of C9 in the killing mechanism may be twofold: to enhance the membrane-labilizing effect of C5b-8 by forming aggregates of this complex; and to form transmembrane protein channels.

Previously, the role of C9 in MAC assembly was thought to be the dimerization of C5b-8 (40). Bound to a small liposome, an individual C5b-8 complex was visualized in an electron microscope as a half-ring connected with the plane of the membrane by a stalk. On addition of C9, the stalk widened, i.e., the space between half-ring and membrane appeared to be spanned by protein. The typical MAC image, which cannot be observed in absence of C9, was therefore interpreted as two fused half-cylinders or as the dimer of C5b-9. This interpretation is essentially correct because the MAC may contain two C5b-8 complexes and 12–16 C9 molecules (41). However, because it is now realized that poly C9 alone produces the ultrastructural image previously thought to be characteristic of the entire MAC (5), the question arises as to the location of C5b-8 within the MAC assembly. Current ultrastructural analyses suggest that C5b-8 is attached to the poly C9 tubule as a structure that is not readily detected by the negative-staining technique (J. Tschopp, H. J. Müller-Eberhard, and E. R. Podack, unpublished observations).

In conclusion, we wish to suggest that C5b-8 polymerizes C9 and that the amphiphilic poly C9 tubule inserts itself into the hydrocarbon core of a target membrane. Thus, we envisage the function of C9 is to provide the poly C9 transmembrane channel of the MAC and the function of C5b-8 is to polymerize C9 and determine the site of channel formation. The structure of the MAC is best described as comprising a poly C9 tubule with one or two C5b-8 attachments.

Summary

Evidence has been presented suggesting that during assembly of the membrane attack complex (MAC) of complement, the C5b-8 complex induces polymerization of

C9. The C9 polymer was detected by sodium dodecyl sulfate (SDS) gel electrophoresis of MAC isolated from complement-lysed erythrocytes. It resembled the previously described polymerized C9 (poly C9) produced from isolated monomeric C9 by prolonged incubation at 37°C in that it was resistant to dissociation by SDS and reducing agents and had an apparent molecular weight of ~1.1 million. The presence of poly C9 in the MAC was further supported by the expression of identical neoantigens by the MAC and poly C9 and by the high C9 content of the MAC relative to its other constituents. Isolated C8 in solution was found to have a single C9-binding site. In mixture, the two proteins formed a reversible equimolar complex that had a sedimentation coefficient of 10.5S. In contrast, a single, cell-bound C5b-8 complex was found to bind up to 12–15 C9 molecules and clusters of C5b-8 bound 6–8 C9 molecules per C8 molecule. In either case, typical ultrastructural membrane lesions were observed, suggesting that the membrane lesion is identical with the tubular poly C9 consisting of 12–16 C9 molecules, and that the MAC can have either the composition (C5b-8)polyC9 or $(C5b\text{-}8)_2$polyC9. When C9 input was restricted so that the molar C9/C8 ratio was ≤ 3, C9-induced aggregates of C5b-8 were observed but virtually no circular membrane lesions were found. We suggest, therefore, that C9, at low dosage, causes cross-linking of multiple C5b-8 complexes within the target membrane and that, at high dosage, C9 is polymerized by C5b-8 to form a transmembrane channel within the MAC assembly. It is primarily the C9 polymer that evokes the ultrastructural image of the MAC or of membrane lesions caused by complement.

We thank Ms. Kerry Pangburn for excellent technical assistance.

References

1. Kolb, W. P., J. A. Haxby, C. M. Arroyave, and H. J. Müller-Eberhard. 1972. Molecular analysis of the membrane attack mechanism of complement. *J. Exp. Med.* **135**:549.
2. Kolb, W. P., and H. J. Müller-Eberhard. 1974. Mode of action of human C9: adsorption of multiple C9 molecules to cell-bound C8. *J. Immunol.* **113**:479.
3. Bhakdi, S., P. Ey, and B. Bhakdi-Lehnen. 1976. Isolation of the terminal complement complex from target sheep erythrocyte membranes. *Biochim. Biophys. Acta.* **419**:445.
4. Podack, E. R., G. Biesecker, W. P. Kolb, and H. J. Müller-Eberhard. 1978. The C5b-6 complex: reaction with C7, C8 and C9. *J. Immunol.* **121**:484.
5. Podack, E. R., and J. Tschopp. 1982. Polymerization of the ninth component of complement (C9): formation of poly C9 with a tubular ultrastructure resembling the membrane attack complex of complement. *Proc. Natl. Acad. Sci. U. S. A.* **79**:574.
6. Podack, E. R., and J. Tschopp. 1982. Characterization of poly C9 and analysis of its pore forming membrane binding site. *Fed. Proc.* **41**:486.
7. Tranum-Jensen, J., S. Bhakdi, B. Bhakdi-Lehnen, O. J. Bjerrum, and V. Speth. 1978. Complement lysis: the ultrastructure and orientation of the C5b-9 complex on target sheep erythrocyte membranes. *Scand. J. Immunol.* **7**:45.
8. Biesecker, G., E. R. Podack, C. A. Halverson, and H. J. Müller-Eberhard. 1979. C5b-9 dimer: isolation from complement lysed cells and ultrastructural identification with complement dependent membrane lesions. *J. Exp Med.* **149**:448.
9. Mayer, M. M. 1972. Mechanism of cytolosis by complement. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2954.
10. Bhakdi, S., and J. Tranum-Jensen. 1978. Molecular nature of the complement lesion. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5655.

11. Podack, E. R., J. Tschopp, and H. J. Müller-Eberhard. 1982. C5b-8 mediated and spontaneous polymerization of C9: formation of C9 polymers with ultrastructural resemblance of the membrane attack complex (MAC). *Mol. Immunol.* In press.
12. Podack, E. R., and H. J. Müller-Eberhard. 1980. Limited proteolysis of C5b-6: functional stability of the degraded complex. *J. Immunol.* **124:**332.
13. Podack, E. R., W. P. Kolb, and H. J. Müller-Eberhard. 1979. Structural similarities between C6 and C7 of human complement. *J. Immunol.* **123:**1071.
14. Kolb, W. P., and H. J. Müller-Eberhard. 1976. The membrane attack mechanism of complement: the three polypeptide chain structure of the eighth component (C8). *J. Exp. Med.* **143:**1131.
15. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large scale isolation of functionally active components of the human complement system. *J. Biol. Chem.* **256:**3995.
16. Biesecker, G., and H. J. Müller-Eberhard. 1980. The ninth component of human complement: purification and physicochemical characterization. *J. Immunol.* **124:**1291.
17. Traker, P. J., and J. C. Speck. 1980. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80:**849.
18. Kolb, W. P., and H. J. Müller-Eberhard. 1975. The membrane attack mechanism of complement: isolation and subunit composition of the C5b-9 complex. *J. Exp. Med.* **141:**724.
19. Laemmli, M. K. 1970. Cleavage of structure proteins during the assembly of the head bacteriophage T4. *Nature (Lond.).* **227:**680.
20. Hadding, U., and H. J. Müller-Eberhard. 1969. The ninth component of human complement: isolation, description and mode of action. *Immunology.* **16:**719.
21. Tschopp, J., and E. R. Podack. 1981. Membranolysis by the ninth component of human complement. *Biochem. Biophys. Res. Commun.* **100:**1409.
22. Kolb, W. P., L. M. Kolb, and J. R. Savary. 1982. Biochemical characterization of the sixth component (C6) of human complement. *Biochemistry.* **21:**294.
23. Kolb, L. M., and W. P. Kolb. 1982. Expression of antithrombin III (ATIII) antigenic determinants by the fluid phase SC5b-9 attack complex of human complement. *Fed. Proc.* **41:**486.
24. Bhakdi, S., and J. Tranum-Jensen. 1981. Molecular weight of the membrane C5b-9 complex of human complement: characterization of the terminal complex as a C5b-9 monomer. *Proc. Natl. Acad. Sci. U. S. A.* **78:**1818.
25. Ware, C. F., R. A. Wetsel, and W. P. Kolb. 1981. Physicochemical characterization of fluid phase (SC5b-9) and membrane derived (MC5b-9) attack complexes of human complement purified by immunoadsorbent affinity chromatography or selective detergent extraction. *Mol. Immunol.* **18:**521.
26. Rommel, F. A., and M. M. Mayer. 1973. Studies of guinea pig complement component C9: reaction kinetics and evidence that lysis of EAC1–8 results from a single membrane lesion caused by one molecule of C9. *J. Immunol.* **110:**637.
27. Stolfi, R. L. 1968. Immune lytic transformation: a state of irreversible damage generated as a result of the reaction of the eighth component in the guinea pig complement system. *J. Immunol.* **100:**46.
28. Harriman, G. R., A. F. Esser, E. R. Podack, A. C. Wunderlich, A. I. Braude, T. F. Lint, and J. G. Curd. 1981. The role of C9 in complement-mediated killing of Neisseria. *J. Immunol.* **127:**2386.
29. Snyderman, R., D. T. Durack, G. A. McCarty, F. E. Ward, and L. Meadows. 1979. Deficiency of the fifth component of complement in human subjects. *Am. J. Med.* **63:**638.
30. Haeney, M. R., R. A. Thompson, J. Faulkner, P. Mackintosh, and A. E. Ball. 1980.

Recurrent bacterial meningitis in patients with genetic defects of terminal complement components. *Clin. Exp. Immunol.* **40:**16.

31. Lee, T. J., A. Schmoyer, R. Snyderman, W. Y. Young, and P. F. Sparling. 1978. Familial deficiencies of the sixth and seventh components of complement associated with bacteremic Neisserial infections. *In* Immunology of *Neisseria gonorrhoeae.* G. F. Brooks, Gotschlich E. C., Holmes K. K., Sawyer, W., and Young, F. E., editors. American Society for Microbiology, Washington, D.C. 204–206.
32. Lee, T. J., P. D. Utsinger, R. Snyderman, W. J. Yount, and P. F. Sparling. 1978. Familial deficiency of the seventh component of complement associated with recurrent bacteremic infections due to Neisseria. *J. Infect. Dis.* **138:**355.
33. Petersen, B. H., J. A. Graham, and G. F. Brooks. 1976. Human deficiency of the eighth component of complement. The requirement of C8 for serum *Neisseria gonorrhoeae* bactericidal activity. *J. Clin. Invest.* **57:**283.
34. Podack, E. R., and H. J. Müller-Eberhard. 1978. Binding of desoxylcholate, phosphatidylcholine vesicles, lipoprotein and of the S-protein to complexes of terminal complement components. *J. Immunol.* **121:**1025.
35. Podack, E. R., G. Biesecker, and H. J. Müller-Eberhard. 1979. Membrane attack complex of complement: generation of high affinity phospholipid binding sites by the fusion of five hydrophilic, plasma proteins. *Proc. Natl. Acad. Sci. U. S. A.* **76:**897.
36. Esser, A. F., W. P. Kolb, E. R. Podack, and H. J. Müller-Eberhard. 1979. Molecular reorganization of lipid bilayers by complement: a possible mechanism for membranolysis. *Proc. Natl. Acad. Sci. U. S. A.* **76:**1410.
37. Schreiber, R. D., D. C. Morrison, E. R. Podack, and H. J. Müller-Eberhard. 1979. Bactericidal activity of the alternative complement pathway generated from eleven isolated plasma proteins. *J. Exp. Med.* **149:**870.
38. Schreiber, R. D., M. K. Pangburn, R. G. Medicus, and H. J. Müller-Eberhard. 1980. Raji cell injury and subsequent lysis by the purified cytolytic alternative pathway to human complement. *Clin. Immunol. Immunopathol.* **15:**384.
39. Podack, E. R., and H. J. Müller-Eberhard. 1981. Complement mediated injury of tumor cells: release of membrane fragments by C5b-9. *Fed. Proc.* **40:**359.
40. Podack, E. R., A. F. Esser, G. Biesecker, and H. J. Müller-Eberhard. 1980. Membrane attack complex of complement: a structural analysis of its assembly. *J. Exp. Med.* **151:**301.
41. Podack, E. R., and H. J. Müller-Eberhard. 1981. Membrane attack complex of complement: evidence for its dimeric structure based on hybrid formation. *J. Biol. Chem.* **256:**3145.