Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7474–7478, December 1982
Immunology

Ultrastructure of the membrane attack complex of complement: Detection of the tetramolecular C9-polymerizing complex C5b-8

[phospholipid vesicles/poly(C9) tubule/supramolecular organization]

JÜRG TSCHOPP, ECKHARD R. PODACK, AND HANS J. MÜLLER-EBERHARD

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

Contributed by Hans J. Müller-Eberhard, August 30, 1982

ABSTRACT **The ultrastructure of the membrane attack complex (MAC) of complement had been described as representing a hollow cylinder of defined dimensions that is composed of the proteins C5b, C6, C7, C8, and C9. After the characteristic cylindrical structure was identified as polymerized C9 [poly(C9)], the question arose as to the ultrastructural identity and topology of the C9-polymerizing complex C5b-8. An electron microscopic analysis of isolated MAC revealed an asymmetry of individual complexes with respect to their length. Whereas the length of one boundary (±SEM) was always 16 ± 1 nm, the length of the other varied between 16 and 32 nm. In contrast, poly(C9), formed spontaneously from isolated C9, had a uniform tubule length (±SEM) of 16 ± 1 nm. On examination of MAC–phospholipid vesicle complexes, an elongated structure was detected that was closely associated with the poly(C9) tubule and that extended 16–18 nm beyond the torus of the tubule and 28–30 nm above the membrane surface. The width of this structure varied depending on its two-dimensional projection in the electron microscope. By using biotinyl C5b-6 in the formation of the MAC and avidin-coated colloidal gold particles for the ultrastructural analysis, this heretofore unrecognized subunit of the MAC could be identified as the tetramolecular C5b-8 complex. Identification also was achieved by using anti-C5 Fab-coated colloidal gold particles. A similar elongated structure of 25 nm length (above the surface of the membrane) was observed on single C5b-8–vesicle complexes. It is concluded that the C5b-8 complex, which catalyzes poly(C9) formation, constitutes a structure of discrete morphology that remains as such identifiable in the fully assembled MAC, in which it is closely associated with the poly(C9) tubule.**

The membrane attack complex (MAC) of complement is a supramolecular organization that is composed of the proteins C5b, C6, C7, C8, and C9 (1–3). The complex assembles spontaneously from its precursor proteins after enzymatic formation of C5b from C5 by C5 convertase (4). During assembly, the complex generates hydrophobic phospholipid binding sites through which it is enabled to bind to cell membranes or synthetic lipid bilayers (5). The MAC impairs membranes and kills cells by disordering the normal lipid bilayer structure and by forming transmembrane channels (6–8).

Electron microscopy afforded the detection of characteristic complement-induced membrane lesions that were originally described as "holes" or "hollow cylinders" (9, 10). The responsible structures were identified later as the MAC itself and two essentially compatible models for MAC architecture were subsequently proposed. The first model described the MAC as a hollow cylinder, 15 nm tall and 10 nm wide, having a wall thickness of 2 nm and an upper torus of 5 nm width (11). All five MAC precursor proteins were thought to be part of the cylindrical structure. The second model also proposed a tubular structure; however, based on molecular weight determinations (12) and on ultrastructural studies (13), the MAC was envisaged to constitute a dimer of C5b-9. It was suggested that the torus of the cylinder contained primarily C5b, C6, and C7 and the thin tubular wall contained primarily C8 and C9, the latter being the MAC subunit most intimately in contact with membrane phospholipids, as photolabeling experiments had shown (14, 15).

Since the original description of the MAC as a compact multimolecular organization, it was known that the MAC contained a multiplicity of C9 molecules relative to the other subunits (1). When it was found that (*i*) isolated C9 by itself has the propensity to form circular polymers [poly(C9)] (16, 17), (*ii*) poly(C9) alone has the ultrastructure previously assigned to the MAC (18), and (*iii*) poly(C9) is an integral constituent of the fully assembled MAC (19), it became apparent that none of the previous models could account for the topology of C5b-8 within the MAC. Because spontaneous poly(C9) formation at 37°C requires many hours (18), whereas C9 polymerization as part of MAC formation occurs within minutes (19, 20), it must be assumed that the tetramolecular C5b-8 complex exercises a catalytic function in the polymerization reaction. The question arose as to the identity and relative topology of the tetramolecular C9-polymerizing complex C5b-8 within the MAC structure. In the following it will be shown that this catalytic unit represents a heretofore unrecognized elongated substructure of the MAC that is closely associated with the poly(C9) tubule.

MATERIALS AND METHODS

Preparation of Poly(C9) and Vesicle Complexes of C5b-8 and C5b-9. Complement proteins were purified as described (21–24). Small unilamellar vesicles were prepared from egg lecithin (Avanti Polar Lipids, Birmingham, AL) by sonication and subsequent Sepharose 4B gel filtration (25). C5b-8–vesicle complexes were prepared by incubation of 2.8 μM vesicles/0.6 μM C5b-6/0.4 μM C7/0.6 μM C8 at 20°C for 5 min. The MAC was generated at 37°C for 10 min from the same reaction mixture, which included, however, 4.8 μM C9. In both cases, C7 was added last. Protein–vesicle complexes were floated through 35% sucrose to remove unbound protein. If the MAC was generated on rabbit erythrocytes, it was isolated in the presence of deoxycholate and purified as published (12). Poly(C9) was prepared at a protein concentration of 1 mg/ml by incubating this solution at 37°C for 64 hr (17).

Conjugation of Avidin or Anti-C5 Fab with Colloidal Gold Particles. Colloidal gold particles (4-nm mean diameter) were prepared as described in detail in ref. 26. In brief, to a neutral solution of 0.01% $HAuCl_4$, sodium borohydrid was added under rapid stirring. For coating the gold particles with avidin, 20 ml of colloidal gold solution (titrated to pH 8 with 0.2 M K_2CO_3) was added to 200 μl of 2 mg of egg white avidin per ml (Sigma),

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: MAC, membrane attack complex of complement.

which had been dialyzed against water. The optimal amount of avidin for stabilization of the gold solution was determined as reported (27). After 10 min, 500 μl of 1% polyethylene glycol (M_r 22,000) (Union Carbide Corp., NY) dissolved in H_2O was added and the avidin–gold conjugate then was concentrated by centrifugation at 35,000 rpm (60 min, 4°C) in a Beckman centrifuge with a 41.1 rotor. The sediment was resuspended in 1.5 ml of Tris-buffered saline (10 mM Tris·HCl/150 mM NaCl, pH 7.4) and stored at 4°C. Before use, 200 μl of the concentrated gold–avidin conjugate was passed over a Sephacryl S-300 column (11 × 0.9 cm) in Tris-buffered saline. The conjugate that eluted after the void volume peak was diluted to a concentration at which the reddish color was barely discernible to the eye. These monodisperse gold particles were subsequently mixed with vesicle-bound C5b-8 and C5b-9 complexes in which the C5b-6 had been modified with biotin (28). Conjugation of anti-C5 Fab fragments with colloidal gold particles was performed in a similar manner. Goat anti-C5 Fab was prepared according to standard procedures (29).

Electron Microscopic Analysis. For electron microscopy, samples were applied to carbon-coated grids that had been rendered hydrophilic by 0.1% (wt/wt) bacitracin. The grids were washed twice with 100 mM NH_4 acetate/50 mM NH_4HCO_3 buffer, pH 7.4, negatively stained with 2% uranyl formate, and examined in a Hitachi 12A electron microscope at 75 kV.

Measurements of the length of the MAC were carried out as follows: Side view images of the MAC exhibiting a rectangular structure were selected and the length of the two parallel boundaries was measured as indicated by the brackets in Fig. 1. Both measurements from an individual complex were used for the analysis regardless of whether they were identical or not. Poly(C9) images were analyzed identically.

RESULTS

Analysis of the Length of MAC Tubules. The MAC extracted and purified from rabbit erythrocytes is hetergeneous in ultrastructural appearance. Fig. 1 shows an electron micrograph of a typical MAC preparation and, for comparison, an electron micrograph of poly(C9). Top views of the MAC and of poly(C9) show typical ring structures that resemble each other. In side views, both the MAC and poly(C9) have a tubular appearance. However, whereas the length of the poly(C9) tubules is uniform, the MAC tubules appear to be asymmetric and of varying length. As seen in Fig. 1*a*, one side of an individual MAC tubule may be twice as long as the opposite side (white arrows and black brackets). The distribution of boundary length of several hundred MAC and poly(C9) side view images is shown in Fig. 2. The tubular length (±SEM) of poly(C9) was found to be uniformly 16 nm ± 1 nm. The length (±SEM) of one of the two sides of the MAC was also 16 ± 1 nm; however, the length of the other side varied between 16 and 32 nm. In almost all MAC images the length of the opposing sides was different.

Ultrastructure of MAC on Phospholipid Vesicles. The MAC was assembled on small unilamellar egg lecithin vesicles by using purified C5b-6, C7, C8, and C9 at a molar ratio of 1:1:1:12. Fig. 3 depicts the ultrastructural appearance of MAC–vesicle complexes. In accordance with earlier studies, side view projections of vesicle-bound MAC are imaged as tubules with inner and outer diameters of 10 and 21 nm, respectively (*a–f*). The tubule projects ≈12 nm above the lipid bilayer. In addition to the tubule that constitutes poly(C9), a 16- to 18-nm long club-like structure is seen, which extends beyond the torus of the poly(C9) tubule (black arrows). This elongated structure, as will be shown below, constitutes the tetramolecular C5b-8 complex. It is ≈5–14 nm wide, depending on whether a lateral or frontal view is inspected. Fig. 3 *b–f* show different views of C5b-8 that were selected because they may represent images of a stepwise 90° rotation of an individual MAC. The projection of C5b-8 changes from a club-like appearance of 5

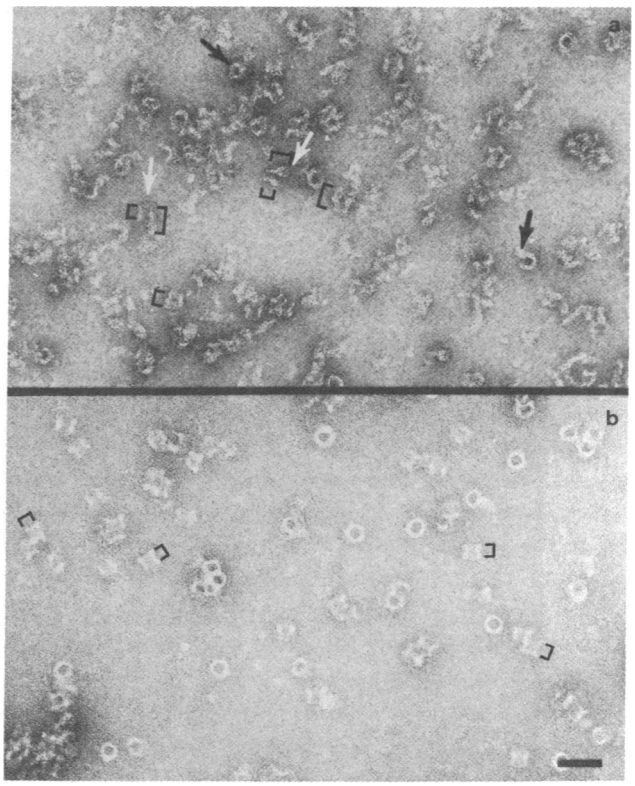

FIG. 1. Ultrastructural comparison of MAC and poly(C9). (*a*) MAC isolated from complement-treated rabbit erythrocytes by extraction with 10% deoxycholate, filtration over a Sepharose 4B column, and sucrose density-gradient ultracentrifugation. The 30S species of the MAC was selected. Black arrows point at top views; the brackets indicate the lengths of side view images. Asymmetrical MAC images with different lengths of the opposing boundaries are indicated by white arrows. (*b*) Poly(C9) shows symmetrical side view images of uniform length. The calibration bar corresponds to 50 nm.

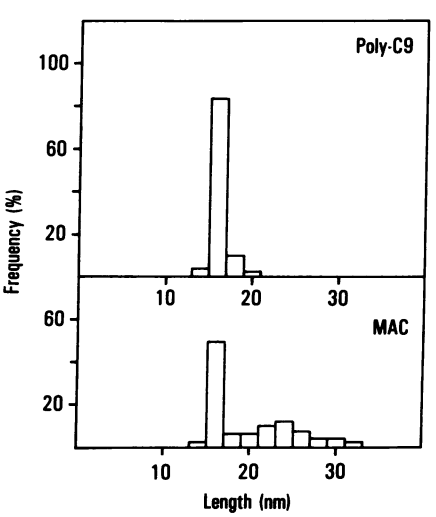

FIG. 2. Histogram of the tubular lengths of poly(C9) and MAC. (*Upper*) The length of 112 poly(C9) tubules was measured. Almost 90% of poly(C9) shows a length of 16 ± 1 nm. (*Lower*) Length distribution of 134 individual MAC images. The 268 measurements (two opposing sides per MAC) exhibit considerable variation.

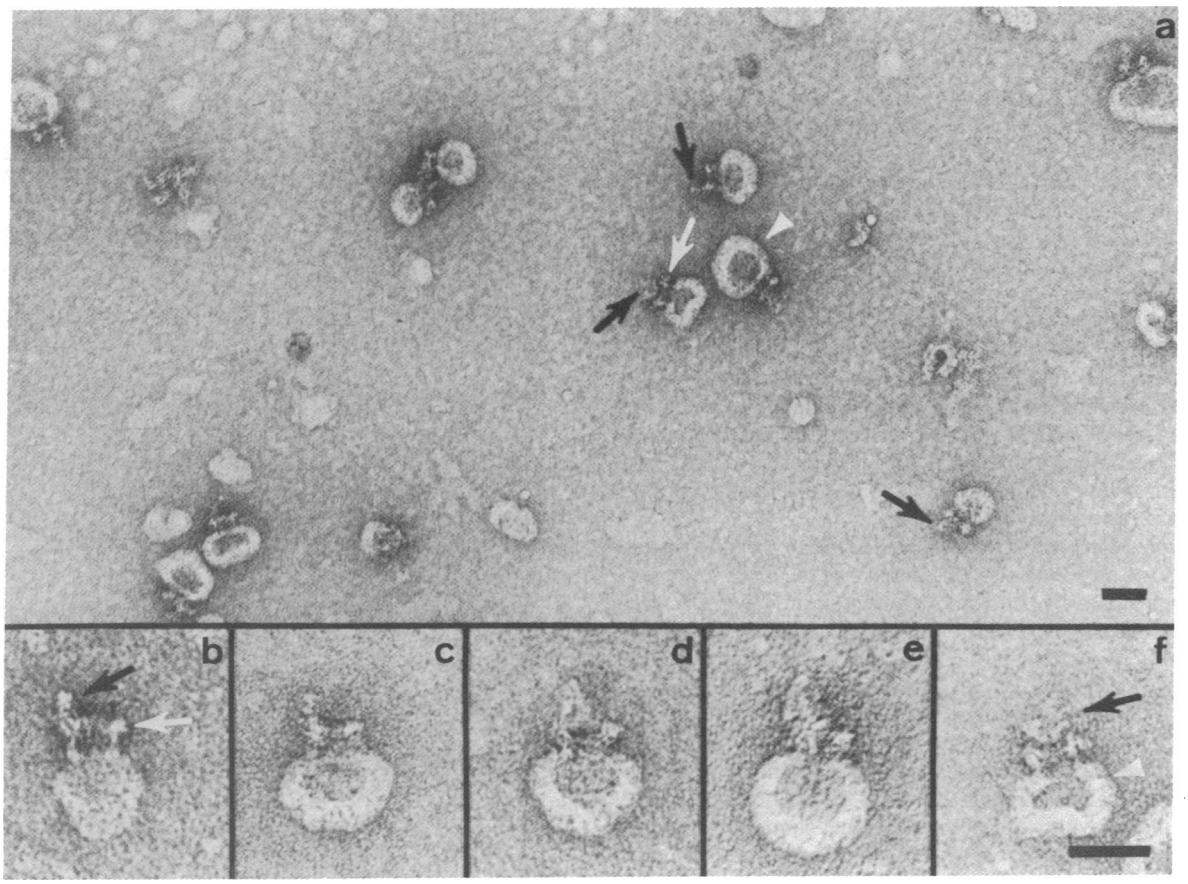

FIG. 3. Ultrastructure of MAC–phospholipid vesicle complexes. (*a*) The majority of the MAC images show, in addition to the cylindrical channel, a 16- to 18-nm club-like structure (black arrows) attached to the poly(C9) tubule (white arrows). (*b–f*) Different views of the C5b-8 complex within the MAC. In a lateral projection (*b* and *c*), the C5b-8 complex is well defined with a width of ≈5 nm; in a frontal view (*d–f*), this complex appears textured with a width of up to 14 nm. The calibration bars correspond to 30 nm.

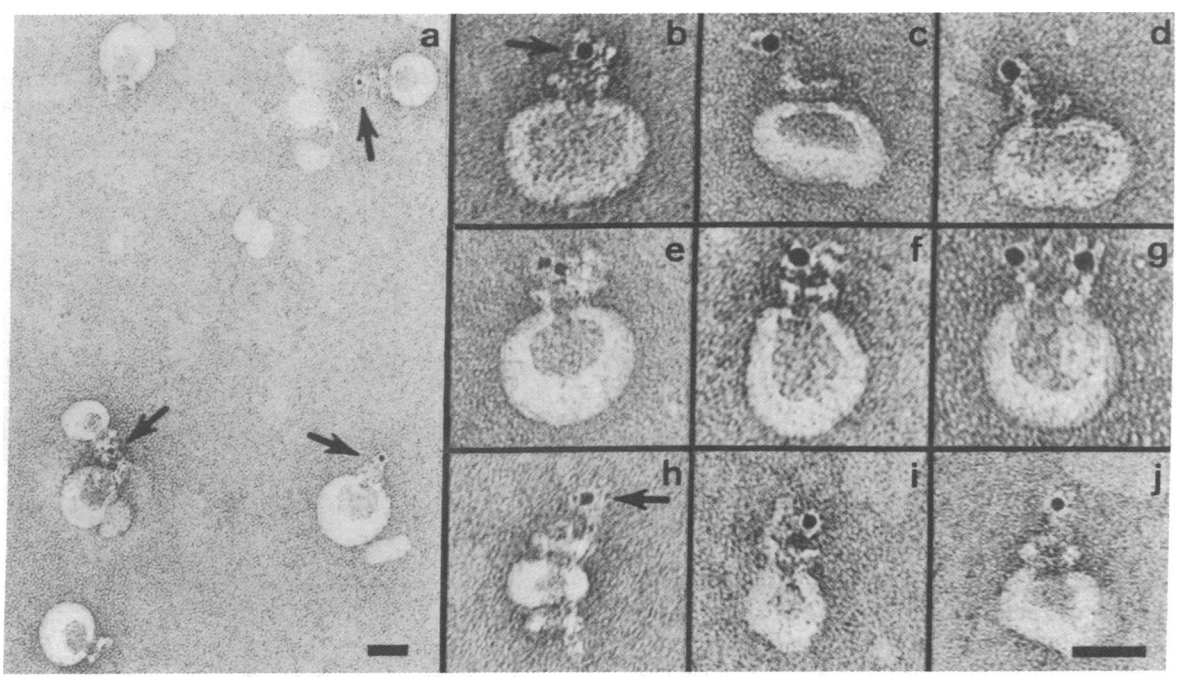

FIG. 4. Identification of the C5b-8 complex within the MAC. Colloidal gold conjugated to either avidin (*b–g*) or anti-C5 Fab fragments (*a* and *h–j*) was used to localize biotinyl C5b-6 or C5b, respectively. The gold probe (black arrows) is consistently found in close proximity to the C5b-8 complex. Generally, only one elongated structure is seen to extend from poly(C9) (*a–f* and *h–j*). A selected MAC image interpretable as comprising one poly(C9) and two C5b-8 complexes is shown in *g*. The calibration bars correspond to 30 nm.

nm width to a hand-shaped structure of ≈14 nm width, both having a length of 28–30 nm above the membrane surface.

Identification of the C5b-8 Complex Within the MAC by Using Colloidal Gold. The C5b-6 complex was modified with biotin and biotinyl C5b-6 was used to initiate MAC assembly on phospholipid vesicles. Avidin adsorbed to colloidal gold then was used to localize biotinyl C5b-6 in the vesicle-bound MAC. As shown in Fig. 4 *a–g*, the gold particle (black arrow) was consistently found 3–20 nm above the poly(C9) torus and obviously was attached to the C5b-8 complex. The same probe location was observed when the gold particles were coated with anti-C5 Fab fragments, thus locating the position of C5 (Fig. 4 *h–j*). In no case were the gold particles observed to attach to the poly(C9) tubules directly.

As in the case of isolated MAC (Fig. 1), most vesicle-bound MAC structures contained only one C5b-8 projection, suggesting that one C5b-8 complex is sufficient to promote circular C9 polymerization (19). Fig. 4*g* shows an exception observed in this study, in which two avidin-coated gold particles are attached to opposite sides of the same MAC structure, suggesting that this particular MAC contained, in addition to poly(C9), two C5b-8 units.

Ultrastructure of C5b-8 in the Absence of Poly(C9). C5b-8–vesicle complexes were generated at a C5b-8-to-vesicle molar ratio of 1:7, which favors the binding of only one C5b-8 complex per vesicle. As shown in Fig. 5, the C5b-8 complex is visualized on vesicles as a 25-nm long and 5- to 14-nm wide club-like structure with a rather polymorphic appearance. Occasionally, the C5b-8 complex was not completely straight—as in Fig. 5 *b*, *c*, and *f*—but was bent in its middle part, as shown in Fig. 5 *d* and *e*. Avidin-coated gold probes recognizing the biotinyl C5b-6 preferentially bound to that end of the C5b-8 complex that is distal to its lipid binding site (Fig. 5*f*). Vesicles bearing one C5b-8 complex were resistant to stain penetration into the vesicle interior. However, when several C5b-8 complexes were attached to one vesicle, they tended to cluster in the membrane (Fig. 5*g*) and allowed stain penetration into the vesicle interior. Within the limits of electron microscopic image interpretation, this observation indicates that a single C5b-8 complex does not suffice to perturb the vesicle membrane.

DISCUSSION

When it became clear that the typical ultrastructural membrane lesion caused by complement (9, 10) represents the image of poly(C9) (17, 18), the question arose as to the ultrastructural identity of C5b-8. This tetramolecular complex has a M_r of ≈550,000 and is closely associated with poly(C9), as analyses of isolated MAC have shown (19), and therefore, should be detectable by electron microscopy. In the present study the complex was detected on C5b-8–vesicle complexes, MAC–vesicle complexes, and on isolated MAC.

Attached to vesicles, C5b-8 projects 25 nm above the surface of the membrane and exhibits a 5–14 nm width. Its structure is club-like or hand-shaped and it may be straight or slightly bent. Because of its polymorphic appearance, its precise ultra-

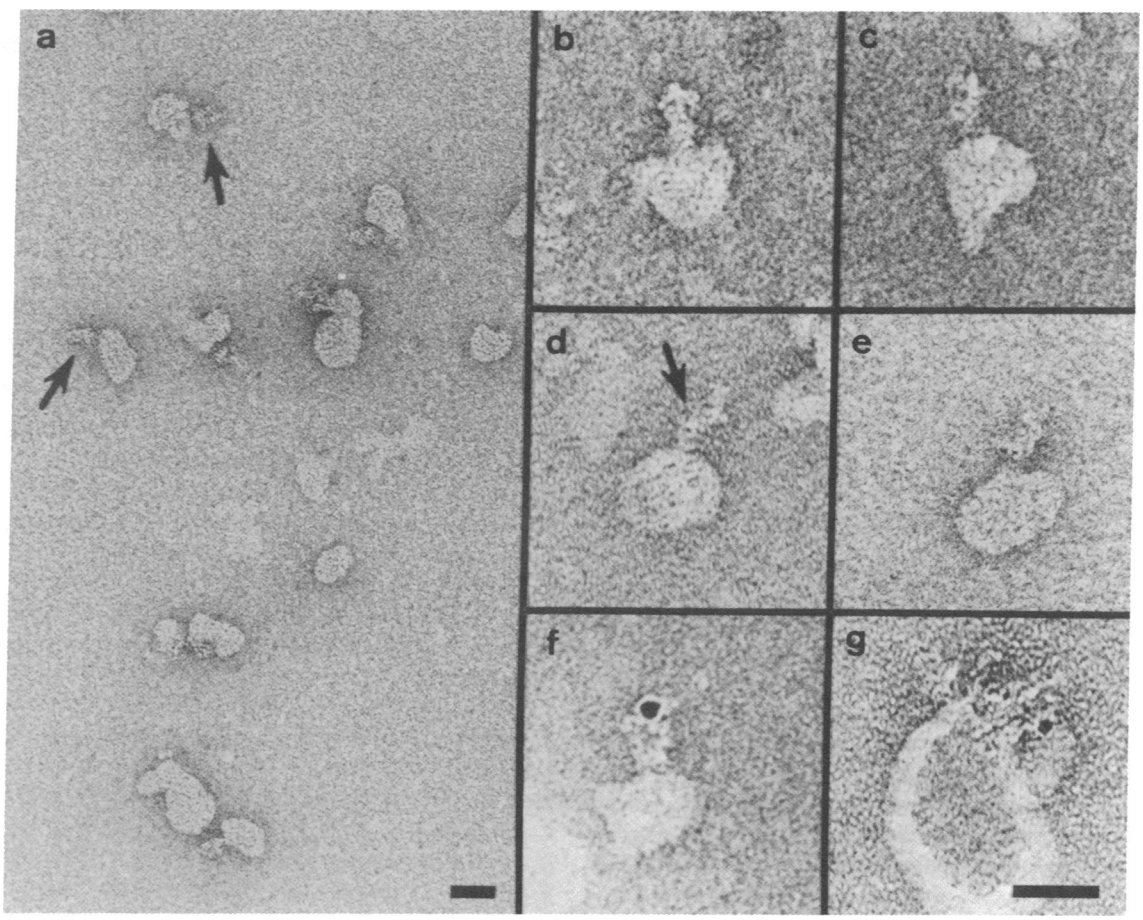

FIG. 5. Ultrastructure of the C5b-8–vesicle complexes. Different views of the C5b-8 complexes are displayed in *a–e*. Structures probably representing frontal views of C5b-8 are shown in *a* (black arrows), whereas the structures seen in *d* and *e* most likely correspond to lateral aspects. The complex may have a bent appearance (*d* and *e*). (*f*) Avidin-coated gold recognizing biotinyl C5b-6 binds to the distal end of the C5b-8 complex. (*g*) Aggregated C5b-8 complexes allow negative stain to diffuse into vesicles. The calibration bars correspond to 30 nm.

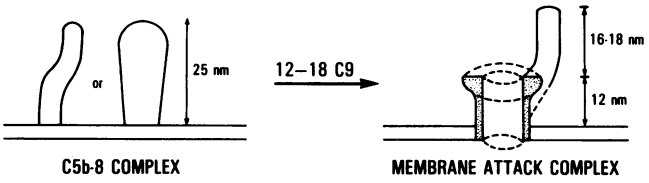

FIG. 6. Schematic representation of the final stage of MAC assembly. Upon binding of C9, C5b-8 catalyzes poly(C9) formation and then can be visualized as an extension of poly(C9). Poly(C9) is responsible exclusively for the tubular structure of the MAC.

structure remains uncertain. C5b-8 is clearly visualized within the MAC organization. It is detectable as a 16- to 18-nm elongated structure that extends beyond the torus of poly(C9) on MAC–vesicle complexes. Therefore, the total length of the structure is 28–30 nm above the surface of the membrane, considering that the visible part of vesicle-bound poly(C9) is 12 nm long. Because C5b-8 without poly(C9) projects only 25 nm above the membrane surface, C9 polymerization by C5b-8 may cause a structural elongation of the C5b-8 complex or its extraction from the membrane. In either case, C5b-8 remains in intimate association with poly(C9) after completion of the polymerization reaction. Fig. 6 depicts a schematic model of C5b-8 and of the fully assembled MAC.

Ultrastructural identification of C5b-8 within the MAC was aided by the use of conjugates of colloidal gold particles. The particles were coated either with avidin to detect biotinyl C5b-6 or with anti-C5 Fab fragments to detect C5b. The gold particles had a diameter of 4 nm and therefore, allowed identification of C5b-6 or C5b with limits of resolution of $\approx$7 nm. The site of attachment of the colloidal gold conjugates strongly suggested that C5b-6 is located in that position of the elongated C5b-8 structure that is distal to the site of binding to the membrane or to poly(C9).

By and large, the electron microscopic analysis detected only one C5b-8 complex within an individual MAC, regardless of whether MAC–vesicle complexes were inspected or MAC was isolated from complement-treated rabbit erythrocytes. This observation supports earlier quantitative studies, which suggested that a single C5b-8 complex is capable of poly(C9) formation (19). However, exceptions have been noted in which two C5b-8 were attached to a single poly(C9) tubule. The latter composition of the MAC may not be exceptional under conditions in which cells are treated with high complement dose and in which the average molar ratio of membrane-bound C9 to C8 is 6 (19).

Micrographs of C5b-8–vesicle complexes revealed that stain penetration into the vesicle interior did not occur if only a single C5b-8 complex was attached to the vesicle. However, stain penetration did occur when multiple complexes were bound. Qualitative as this observation is, it may be pertinent to the mechanism of cell lysis by C5b-8 in absence of C9.

Although membrane-bound C5b-8 has been viewed previously by electron microscopy (13, 30), it had escaped detection as a structural subunit of the MAC. On the basis of the present study, it is now possible to identify C5b-8 in many of the earlier published electron micrographs of the MAC (10, 13, 28, 30–32).

We thank Dr. M. C. Chang for the preparation of the colloidal gold and Kerry Pangburn and Sara Patthi for excellent technical assistance. This is publication no. 2806 from the Research Institute of Scripps Clinic. This research was supported by National Institutes of Health Grants AI 17354, AI 18525, CA 27489, and HL 16411. J.T. was supported by the Swiss National Science Foundation. E.R.P. is an Established Investigator of the American Heart Association (no. 79-149). H.J.M.-E. is the Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic.

1. Kolb, W. P., Haxby, J. A., Arroyave, C. M. & Müller-Eberhard, H. J. (1972) *J. Exp. Med.* **135**, 549–566.
2. Kolb, W. P. & Müller-Eberhard, H. J. (1973) *J. Exp. Med.* **138**, 438–451.
3. Bhakdi, S., Ey, P. & Bhakdi-Lehnen, B. (1976) *Biochim. Biophys. Acta* **419**, 445–451.
4. Kolb, W. P. & Müller-Eberhard, H. J. (1975) *J. Exp. Med.* **141**, 724–735.
5. Podack, E. R. & Müller-Eberhard, H. J. (1978) *J. Immunol.* **121**, 1025–1030.
6. Esser, A. F., Kolb, W. P., Podack, E. R. & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1410–1414.
7. Mayer, M. M. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2954–2958.
8. Podack, E. R., Müller-Eberhard, H. J., Horst, H. & Hoppe, M. (1982) *J. Immunol.* **128**, 2353–2357.
9. Borsos, T., Dourmashkin, R. R. & Humphrey, J. H. (1964) *Nature (London)* **202**, 251–252.
10. Humphrey, J. H. & Dourmashkin, R. R. (1969) *Adv. Immunol.* **11**, 75–115.
11. Tranum-Jensen, J., Bhakdi, S., Bhakdi-Lehnen, B., Bjerrum, O. J. & Speth, V. (1978) *Scand. J. Immunol.* **7**, 45–56.
12. Biesecker, G., Podack, E. R., Halverson, C. A. & Müller-Eberhard, H. J. (1979) *J. Exp. Med.* **149**, 448–458.
13. Podack, E. R., Esser, A. F., Biesecker, G. & Müller-Eberhard, H. J. (1980) *J. Exp. Med.* **151**, 301–313.
14. Podack, E. R., Stoffel, W., Esser, A. F. & Müller-Eberhard, H. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4544–4548.
15. Hu, V. W., Esser, A. F., Podack, E. R. & Wisnieski, B. J. (1981) *J. Immunol.* **127**, 380–386.
16. Tschopp, J. & Podack, E. R. (1981) *Biochem. Biophys. Res. Commun.* **16**, 1409–1414.
17. Podack, E. R. & Tschopp, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 574–578.
18. Tschopp, J., Müller-Eberhard, H. J. & Podack, E. R. (1982) *Nature (London)* **298**, 534–538.
19. Podack, E. R., Tschopp, J. & Müller-Eberhard, H. J. (1982) *J. Exp. Med.* **156**, 268–282.
20. Tschopp, J., Podack, E. R. & Müller-Eberhard, H. J. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 486 (abstr.).
21. Podack, E. R. & Müller-Eberhard, H. J. (1980) *J. Immunol.* **124**, 332–336.
22. Podack, E. R., Kolb, W. P., Esser, A. F. & Müller-Eberhard, H. J. (1979) *J. Immunol.* **123**, 1071–1077.
23. Kolb, W. P. & Müller-Eberhard, H. J. (1976) *J. Exp. Med.* **143**, 1131–1139.
24. Biesecker, G. & Müller-Eberhard, H. J. (1980) *J. Immunol.* **124**, 1291–1296.
25. Mason, J. T. & Hung, C. (1978) *Ann. N.Y. Acad. Sci.* **308**, 29–49.
26. Chang, M. C., Ming, C., Preissner, K. T. & Tschopp, J. (1982) *J. Cell. Biol.*, in press.
27. Horisberger, M., Rosset, J. & Bauer, H. (1975) *Experientia* **31**, 1147–1148.
28. Podack, E. R. & Müller-Eberhard, H. J. (1981) *J. Biol. Chem.* **256**, 3145–3148.
29. Sundsmo, J. S. & Müller-Eberhard, H. J. (1979) *J. Immunol.* **122**, 2371–2378.
30. Dourmashkin, R. R. (1978) *Immunology* **35**, 205–212.
31. Mayer, M. M. (1978) *Harvey Lect.* **72**, 139–193.
32. Bhakdi, S. & Tranum-Jensen, J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5655–5659.