C5b-9 DIMER: ISOLATION FROM COMPLEMENT LYSED CELLS AND ULTRASTRUCTURAL IDENTIFICATION WITH COMPLEMENT-DEPENDENT MEMBRANE LESIONS*

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The exact nature of the ultrastructural membrane lesions (1) caused by the membrane attack complex (C5b-9) (2-4) of complement has been unknown. Visualized by the negative staining technique, the lesions exhibit a dark central area which is surrounded by a ring having an external diameter of approximately 200 Å (5). The occurrence of the lesions is dependent upon the presence on the target membrane of all five proteins of the membrane attack complex: C5b, C6, C7, C8, and C9 (6-8). Ferritin-labeled antibodies to C5 and C8 (M. J. Polley, unpublished observations) and to C9 (9), applied to complement lysed cells, were found to localize in the immediate vicinity of the complement produced lesions. These observations suggested that the proteins of the C5b-9 complex have a close topological relationship to the ultrastructural lesions.

It has previously been shown that membrane bound C5b-9 has a fluid phase equivalent. It is formed upon complement activation in serum (3, 4) and contains an additional protein, called S-protein, which is an inhibitor of forming C5b-9 (10). The SC5b-9 complex was found to have a mol wt of 1.04 × 10^6 daltons and, as will be shown below, an ultrastructure that is apparently unrelated to the structure of the membrane lesions. In contrast, the C5b-9 complex extracted from complement lysed cells was reported to have the ultrastructure of a hollow cylinder and the electron microscopic appearance of complement produced membrane lesions (11).

We wish to report that the membrane attack complex extracted from complement lysed cells by deoxycholate constitutes the dimer of C5b-9 and that it is the dimeric form of the complex that, by itself or incorporated into lipid bilayers, evokes the ring shaped image of the typical complement lesions. The term membrane attack complex, (MAC), will therefore be applied only to the complex having the structural formula (C5b-9)₂.

Materials and Methods

Isolated Proteins. C5b-6 (12), C6 and C7 (13), C8 (14) and SC5b-9 (4) were purified according to published methods. C9 was purified according to the method of Biesecker and Müller-}

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Abbreviations used in this paper: MAC, membrane attack complex; DOC, deoxycholate; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetra-acetic acid.
Proteins were radiolabeled with iodine by the chloramine T method (15) and radioactivity measured using a Packard auto-gamma scintillation counter.

Serum and Erythrocytes. Rabbit erythrocytes were prepared from fresh rabbit blood drawn into ethylenediamine tetra-acetic acid (EDTA) (final concentration 0.01 M). The cells were pelleted by centrifugation for 10 min at 2,000 rpm (1,100 g, International model PR-2, International Equipment Co., Needham Hts., Mass.), washed four times with veronal-buffered saline containing 5 mM EDTA, then washed three times and resuspended with veronal-buffered saline containing 1% (wt/vol) gelatin plus 0.5 mM MgCl₂ and 0.15 mM CaCl₂.

Freshly drawn human blood was allowed to clot for 2 h at 37°C and 2 h at 0°C. The clot was removed by centrifugation for 20 min at 2,000 rpm. After the addition of ¹²⁵I-C₉ (50 ng/ml) the serum was centrifuged for 30 min at 12,000 rpm (Sorvall, HB4 rotor) to remove any aggregated C₉.

Formation and Purification of MAC. Serum was mixed with rabbit erythrocytes at a concentration of 2.5 to 5 × 10⁸ cells/ml of serum either by suspending packed cells or mixing the serum 1:1 with cells suspended in veronal-buffered saline containing gelatin plus calcium and magnesium. The cells were lysed by incubation at 37°C for 20 min. The cell membranes were pelleted by centrifugation at 12,000 rpm for 30 min (Sorvall, HB4 rotor, Sorvall-Du Pont Co., Biomedical Div., Newtown, Conn.) and washed twice with 5 mM EDTA, pH 8.0, 4°C. To the packed white membranes was added 15% deoxycholate (DOC) (Sigma Chemical Co., St. Louis, Mo.), 50 mM Tris-acetate, pH 8.9, to a final DOC concentration of 10% and the complex was extracted for 2 h at room temperature. The extract was then centrifuged at 40,000 rpm (100,000 g) for 1 h (Beckman L-5-65, SW 50.1 rotor, Beckman Instruments, Inc., Fullerton, Calif.). The detergent extracted MAC was purified by gel filtration in the presence of 2% DOC on BioGel A15m. The complex containing fractions were pooled on the basis of radioactivity and concentrated by Amicon ultrafiltration, Amicon Corp., Lexington, Mass., (PM 30 membrane). The protein sample was then dialyzed against 2% DOC, 50 mM Tris-acetate, pH 8.6. Rabbit cells were also lysed with guinea pig serum containing ¹²⁵I-C₉ (human) and the guinea pig-MAC extracted as described above for human MAC. Human MAC was extracted from sheep erythrocyte membranes prepared similarly to the rabbit membranes.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis was performed according to the method of Weber and Osborn (16). Because high concentrations of DOC interfere with SDS binding to proteins, the samples were dialyzed overnight at 37°C against 2% SDS, 6 M urea before electrophoresis. Phosphate analysis was performed according to the method of Bartlett (17).

Sucrose Density Gradient Ultracentrifugation. Samples were centrifuged on 5-ml linear gradients of 10-50% (wt/vol) sucrose, dissolved with 50 mM Tris-acetate, pH 8.6. Some of the gradients also contained 1% DOC. Centrifugation was carried out in a Beckman L-5-65 ultracentrifuge (SW 50.1 rotor) and fractions were collected from the bottom via a needle punched through the tube or from the top using an Auto Densi-flow IIC (Buchler Instruments Div., Searle Diagnostics, Inc., Fort Lee, N. J.).

Electron Microscopy. Negatively stained samples were deposited onto parlodion and carbon coated 400-mesh copper grids made hydrophilic by placing a drop of 0.01% (wt/vol) bacitracin on each and drawing off excess fluid with filter paper. 5 µl of the protein sample (approximately 0.1 mg/ml) was applied and after 10-30 s, excess sample was removed. 5 µl of 2% phosphotungstic acid, pH 7.0, or uranyl oxalate, pH 6.8 (18), in distilled water was added. The stain was drawn off immediately in the case of phosphotungstic acid, and after 30 s with uranyl oxalate, and the grid was allowed to air dry. Grids were examined in a Hitachi 12A electron microscope operated at 75 kV and a direct magnification of 100,000, with a 500-µm second condenser aperture and a 20-µm objective aperture. The bright-field image was recorded on Kodak 4463 electron image film.

Formation of MAC with Purified Proteins. The C₅b-₉ complex was also formed from purified components by reactive lysis (19). Purified C₅b-₆, C₈ and C₉ were added to mixed DOC-phospholipid micelles and incubated for 10 min at 37°C. Then, C₇ was added and the mixture incubated for a further 30 min to form the C₅b-₉ complex. The complex was extracted with 10% DOC and analyzed on sucrose density gradient ultracentrifugation.

Reincorporation of MAC into Phospholipid Liposomes. The MAC was reincorporated into phos-
phospholipid liposomes formed by the method of Brunner et al. (20). 1 mg of dried egg lecithin was dissolved in 100 μl 30 mM DOC, 0.9 M NaCl, 20 mM Tris-acetate, pH 8.6. 200 μl of MAC (0.4 mg/ml; 2% DOC) was added and the sample was gel filtered at 1 ml/h on Sephadex G50 at 4°C (1.5 × 20 cm; 0.9 M NaCl, 20 mM Tris-acetate, pH 8.6) to remove the DOC. The liposomes eluting in the void volume were examined by electron microscopy.

Results

Characterization of the Membrane Attack Complex as a C5b-9 Dimer. MAC formation was induced on the surface of unsensitized rabbit erythrocytes. This procedure produced four to five times the amount of MAC than formation of MAC on antibody-coated sheep erythrocytes. The MAC could not be eluted from the membranes at high or low salt concentration, high pH, or by 0.2 M EDTA, treatments which suffice to remove peripheral membrane proteins (21). The nonionic detergents Triton X-100 and Tween 80 at a concentration of 1% were ineffective in extracting MAC (19).

The complex was separated from other proteins by gel filtration on BioGel A15m (Fig. 1). It eluted as a single symmetrical, although broad peak in the included volume of the column when gel filtration was performed in 2% DOC. Lowering the DOC concentration, although not below the critical micelle concentration, resulted in aggregation of some of the MAC, which then eluted in the void column. MAC subjected to molecular sieve chromatography in 1% Triton X-100 was completely aggregated.

Analysis of the purified, unreduced MAC by SDS polyacrylamide gel electrophoresis allowed identification of its subunits as C5b, C6, C7, C8α-γ, C9, and C8β (Fig. 2). Except for absence of the S-protein, the pattern was qualitatively and quantitatively identical to that of SC5b-9, including the molar ratios of the subcomponents relative to C7 (4, 22). Fig. 3 shows the sedimentation behavior of the purified MAC and of SC5b-9 upon 10-50% sucrose density gradient ultracentrifugation. The sedimentation coefficient of the MAC was 33.5 S compared to 23 S for SC5b-9 (3). Fig. 4 shows the purified MAC and SC5b-9 upon gel filtration through Sepharose 4B. The diffusion coefficient of the MAC was found to be $1.79 \times 10^{-7}$ cm$^2$/s for SC5b-9 (3). On the basis of the s- and D-values, the mol wt of the
Figure 2. Subunit composition of MAC compared to that of SC5b-9. The complexes were subjected to SDS polyacrylamide gel electrophoresis in unreduced form. The analyses were performed on different days under slightly different conditions. The protein bands were identified as indicated. Except for the S-protein which is absent from the MAC, the subunit compositions of both complexes are identical. Degradation of C5b to C5c is more extensive in the MAC than in SC5b-9.

MAC was calculated to be $1.7 \times 10^6$ daltons and that of SC5b-9 $1.0 \times 10^6$ daltons, assuming a partial specific volume ($\bar{\nu}$) of 0.73 ml/g for both complexes (Table I). No phospholipid was detected in the purified MAC by phosphate analysis.

Human MAC extracted from sheep erythrocytes and guinea pig MAC extracted from rabbit erythrocyte membranes also had sedimentation coefficients of 33.5 S, indicating that the size of the MAC is independent of the complement source and the species of the target membrane used. The 33.5 S MAC was also obtained when the complex was assembled using the purified components C5b-6, C7, C8, and C9 in presence of mixed phospholipid-DOC micelles and then extracted from the micelles with excess DOC.

By investigating the effect of detergent binding on the size of the extracted complex, it was found that the observed difference in size between the MAC and SC5b-9 was not a result of DOC binding. The purified MAC was added to serum and DOC was removed by dialysis. The serum treated, detergent-free MAC sedimented upon sucrose density gradient ultracentrifugation as a nonaggregated 33.5 S component. Analysis of the complex by Ouchterlony test and SDS polyacrylamide gel electrophoresis showed the complex to contain, in addition to C5b to C9, also the S-protein. Thus, during dialysis DOC was replaced by S-protein. The fact that the S-protein-MAC complex had an s-rate of 33.5 S instead of 23 S shows that DOC binding was not responsible for the large size of the DOC extracted MAC. It also shows that the MAC was not dissociated to the C5b-9 monomer by binding of the S-protein. Neither was the MAC dissociated by reduction (10 mM dithiothreitol for 1 h at 37°C) or
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FIG. 3. Determination of sedimentation coefficient of MAC by ultracentrifugation in 10-50% sucrose density gradients. The buffer contained 1% DOC. $^{125}$I-MAC sedimented with $s = 33.5$ S and $^{125}$I-SC5b-9 with $s = 23$ S. The latter had a faster sedimenting shoulder which was not seen when SC5b-9 was analyzed in absence of MAC. The lighter $^{125}$I-labeled material represents some dissociated S-protein.

Identification of the Complement Membrane Lesion as C5b-9 Dimer. Electron microscopic examination of the extracted MAC yielded images that are identical with the appearance of the characteristic membrane lesions (1, 5) caused by complement (Fig. 5). The ring-like image has an external diameter of 200 Å. The other image, which may represent a side view, consists of two adjacent, elongated wedges with their long axis measuring 240 Å when stained with uranyl oxalate. In contrast, the SC5b-9 complex appears as a single wedge with its long axis measuring 320 Å (Fig. 6). Fig. 7 shows that the extracted MAC was capable of reincorporation into phospholipid bilayer liposomes. The MAC was mixed with egg lecithin-DOC micelles and the DOC was separated from the lipid vesicles by gel filtration. Various aspects of the reincorporated MAC are seen which appear as typical complement-dependent membrane lesions.

Discussion

The present study has shown that (a) the MAC extracted from complement lysed cells constitutes the dimer of C5b-9, (b) the typical complement-dependent membrane proteolysis with trypsin (100 μg/ml, 1 h at room temperature).

The 33.5 S MAC was dissociated to the 23S C5b-9 monomer by incubation in 2% DOC, 0.1% SDS for 1 h at room temperature. Although SDS alone is denaturing, the mixed DOC-SDS micelles were not, as evidenced by the fact that none of the radiolabeled C9 was dissociated from the complex.
lesion is identical with this dimer and (c) the isolated MAC can be reincorporated into phospholipid bilayers.

It was previously proposed that the cytolytic MAC is the membrane bound analogue of the fluid phase SC5b-9 complex (3). SC5b-9 has a mol wt of 1,040,000 daltons (3) and the C5b-9 portion, which was thought to correspond to the MAC, has a mol wt of 800,000 daltons. In contrast, the MAC extracted with DOC from complement lysed cells was approximately twice as large: 1.7 million daltons. Because the subunit composition of the extracted MAC was identical to that of SC5b-9 except for lack of the S-protein, we concluded that the MAC represents (C5b-9)_2. The possibility was considered that the dimer was produced during extraction with DOC. It was ruled out, however, by the observation that the dimer has the typical ultrastructural appearance of a complement produced membrane lesion, while the ultrastructure of the SC5b-9 complex bears no apparent relationship to the lesion. Furthermore, when the isolated dimer was mixed with DOC-phospholipid micelles
and DOC was subsequently removed, the resultant phospholipid liposomes exhibited characteristic complement lesions on their surface.

It is proposed therefore that the structural formula of the MAC of human complement is \( (C_{5b}, C_6, C_7, C_8, C_{9b})_2 \). Dimerization permits structural symmetry within the complex and this may be an essential prerequisite for attaining the ring-like morphology visualized upon examination of the isolated MAC. Whether the MAC has the geometry of a hollow cylinder, as has been proposed by others (11), or a different geometry remains to be elaborated in the future.

That the appearance of the typical membrane lesions depends on C9 has been established (7, 8, 23). Taken together with the present identification of lesions as C5b-9 dimers, these observations suggest that dimer formation is induced by binding of C9 to C5b-8. If dimer formation occurs at the C9 stage of MAC assembly, then the C5b-9 monomers should have a certain degree of freedom for lateral movement within the target membrane. Freedom of movement is likely because the forming MAC acts on the lipid compartment of membranes and the lesions tend to occur in clusters. Whether dimerization can occur at an earlier stage of MAC assembly is not known,

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**Fig. 5.** Ultrastructural analysis of the MAC. Electron micrographs of the isolated MAC stained with phosphotungstic acid (left) at 250,000:1 (upper) and 500,000:1 (lower) and stained with uranyl oxalate at 500,000:1 (right). Visualized are ring-like structures closely resembling those seen on complement lysed cells. In addition, side views are seen which appear as two adjacent wedges.
since DOC extracted C5b-7 and C5b-8 complexes invariably form large aggregates. It should be emphasized that functional membrane lesions are already generated at the C5b-8 stage of MAC assembly (24, 25) when ultrastructural lesions are not yet detectable. However, the rate of cytolysis is greatly enhanced with attachment of C9 and C5b-9 dimer formation. The two-step development of cytolytic activity may be related to the increasing phospholipid binding capacity of the forming MAC. It was found recently in this laboratory that C5b-7, C5b-8 and (C5b-9)$_2$ are capable of binding respectively 400, 800, and 1,400 mol of phospholipid per mole of complex (26). The degree of molecular reorganization of membrane lipids effected by these complexes may determine the extent of membrane impairment: the C5b-9 dimer causes extensive transmembrane flux of small ions and water, the C5b-8 complex allows minimal transmembrane flux and the C5b-7 complex is unable to cause such a membrane leak.

Summary
The membrane attack complex (MAC) of complement was extracted from the membranes of cells lysed by human complement and its properties were compared
with those of the fluid phase complex SC5b-9. Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunochemical analysis, the two isolated complexes had identical subunit compositions, except that the MAC lacked the S-protein. The sedimentation coefficient and molecular weight of the extracted and isolated MAC were, respectively, 33.5 S and $1.7 \times 10^6$ daltons, compared to 23 S and $1.0 \times 10^6$ dalton for SC5b-9. Because the molecular weight of the MAC is approximately two times greater than that of C5b-9 (800,000 daltons), the MAC is considered the dimer of C5b-9. Under specified conditions, the 33.5 S dimer could be converted to the 23 S monomer without dissociation of subunits. The MAC had the electron microscopic appearance and dimensions that are characteristic for the complement produced ultrastructural membrane lesions. SC5b-9 had a different ultrastructure that is dissimilar to the morphology of the lesions. The isolated MAC could be reincorporated into phospholipid bilayers and assumed on the surface of the resultant lipid vesicles the orientation and appearance of typical complement lesions.

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