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Molecular composition of the terminal membrane and fluid-phase C5b–9 complexes of rabbit complement

Absence of disulphide-bonded C9 dimers in the membrane complex

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The terminal membrane C5b–9(m) and fluid-phase SC5b–9 complexes of rabbit complement were isolated from target sheep erythrocyte membranes and from inulin-activated rabbit serum respectively. In the electron microscope, rabbit C5b–9(m) was observed as a hollow protein cylinder, a structure identical with that of human C5b–9(m). Monodispersed rabbit C5b–9(m) exhibited an apparent sedimentation coefficient of 29S in deoxycholate-containing sucrose density gradients, corresponding to a composite protein–detergent molecular-weight of approx. 1.4×10^6. Protein subunits corresponding to human C5b–C9 were found on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. By densitometry, there were consistently six molecules of monomeric C9 present for each monomeric C5b–8 complex. Fluid-phase rabbit SC5b–9 was a hydrophilic 23S macromolecule that differed in subunit composition from its membrane counterpart in that it contained S-protein and only two to three molecules of C9 per monomer complex. The data are in accord with the previous report on human C5b–9 that C5b–9(m) contains more C9 molecules than SC5b–9 [Ware & Kolb (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6426–6430]. They corroborate the previous molecular-weight estimate of approx. 10^6 for C5b–9(m) and thus support the concept that the fully assembled, unit lesion of complement is a C5b–9 monomer [Bhakdi & Tranum-Jensen (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1818–1822]. They also show that C9 dimer formation is not required for assembly of the rabbit C5b–9(m) protein cylinder, or for expression of its membrane-damaging function.

Complement damages membranes through the binding of the five terminal components C5–C9 to the lipid bilayer with the formation of a macromolecular C5b–9(m) complex (Thompson & Lachmann, 1970; Lachmann & Thompson, 1970; Kolb *et al.*, 1972; Kolb & Müller-Eberhard, 1973, 1974). This process involves conformational changes leading to exposure of lipid-binding regions that allow for partial protein insertion into the membrane. The terminal C5b–9(m) complex possesses the structure of a hollow protein cylinder, and functional as well as ultrastructural studies support the concept that membrane damage ensues through generation of transmembrane channels walled by the inserted protein cylinders (Mayer, 1972; Michaels *et al.*, 1976; Giavedoni *et al.*, 1979; Mayer *et al.*, 1981; Bhakdi & Tranum-Jensen, 1978; Tranum-Jensen *et al.*, 1978).

Abbreviations used: C5b–9(m), membrane C5b–9 complement complex; SDS, sodium dodecyl sulphate.

The question regarding the molecular composition of C5b–9(m) is surrounded by controversy. Careful initial studies by Kolb *et al.* (1972) indicated that each membrane-bound C5b–8 monomer complex was capable of binding a finite number of six C9 molecules. A structural formula of $(C5b–8)_1C9_6$ was therefore tentatively proposed before the actual isolation of the protein. The theoretical molecular weight of such a protein complex would be approx. 10^6 (Kolb *et al.*, 1972).

Subsequently, the C5b–9(m) complex of human complement was isolated and its subunit composition determined by SDS/polyacrylamide-gel electrophoresis. By densitometry, two to three monomeric C9 molecules were found per molecule of C5b–8 monomer (Bhakdi *et al.*, 1976; Biesecker *et al.*, 1979), and the protein molecular weight of

each C5b–9(m) monomer was thus first believed to be in the order of 800000. This tentative value was also obtained from a comparison with the composition of fluid-phase SC5b–9. There is no disagreement on the composition of the latter (Kolb & Müller-Eberhard, 1975; Bhakdi *et al.*, 1979); SC5b–9 is a water-soluble 23S protein composed of one C5b–8 monomer plus two to three molecules of C9 and two to three molecules of S-protein. Its molecular weight is slightly over 10^6. Apart from the additional presence of S-protein, the subunit composition of SC5b–9 appeared identical with that of C5b–9(m). Subtracting the molecular weight of S-protein, a C5b–9(m) monomer would therefore be expected to have a molecular weight of approx. 800000.

Data were then presented by Biesecker *et al.* (1979) that appeared to exclude such a monomer nature of the C5b–9(m) cylinder. Those authors reported a sedimentation coefficient of 33.5S and a molecular weight of 1.7×10^6 for the complex in deoxycholate solution, and concluded that each membrane-attached protein cylinder must therefore represent a dimer with the structural formula $(C5b–8)_2C9_6$ (Biesecker *et al.*, 1979). Podack *et al.* (1980) and Podack & Müller-Eberhard (1981) later provided additional data that appeared to support this contention.

However, a reinvestigation led us to question the validity of the dimer concept. In our experience, detergent-solubilized C5b–9(m) was present in various states of oligomeric self-association, whereby the possibility that this represented an experimental artefact arising after or during the procedure of membrane protein isolation and purification was not excluded (Bhakdi & Tranum-Jensen, 1981). Truly monodispersed C5b–9(m) cylinders sedimented as 26S rather than as 33.5S entities in sucrose density gradients containing Triton X-100 + deoxycholate. After correcting for bound detergent, we arrived at a molecular-weight estimate of approx. 10^6 for each C5b–9(m) protein cylinder. We therefore concluded that the unit complement lesion is a C5b–9 monomer and not a dimer. This concept received support from earlier structural (Bhakdi & Tranum-Jensen, 1979) as well as from more recent functional (Ramm *et al.*, 1982) and biochemical studies (Bhakdi & Tranum-Jensen, 1982*a*) on the complement lesion.

Yet the relatively high molecular-weight estimate obtained in our studies remained puzzling. New light was recently shed on this issue when Ware & Kolb (1981) showed that each C5b–9(m) monomer contained one disulphide-bonded C9 dimer additional to the C9 monomers. The C9 dimer had previously been erroneously assumed to represent C5c (Bhakdi *et al.*, 1976; Biesecker *et al.*, 1979). Ware & Kolb (1981) thus concluded that membrane C5b–9 in effect contains a higher number of C9 molecules than does SC5b–9. They also thought that C9 dimer formation may be of relevance for the expression of lytic C5b–9 function.

Should C5b–9(m) indeed harbour more C9 than SC5b–9, this would satisfactorily explain its relatively high molecular weight. In an attempt to corroborate these data, we have now extended our studies to the rabbit terminal C5b–9 complexes. We present evidence that the unit lesion of rabbit complement is a protein cylinder of the same basic structure as human C5b–9(m). The cylinders exhibit an apparent sedimentation coefficient of 29S in deoxycholate solution, corresponding to a composite protein–detergent molecular weight of approx. 1.4×10^6. Analysis of the protein subunits contained within the complex confirm that fully assembled C5b–9(m) complexes contain more C9 molecules than fluid-phase SC5b–9. They support the notion that each protein cylinder is a C5b–9 monomer of mol.wt. $(1–1.1) \times 10^6$, with the structural formula $(C5b–8)_1C9_6$, and also indicate that C9 dimer formation is not an essential event in the generation of the terminal rabbit complex.

Materials and methods

Isolation of rabbit C5b–9(m)

A 10% (v/v) suspension of sheep erythrocytes sensitized with antibody (Ambozeptor, titre 1:6000; Behringwerke, Marburg, Germany) was prepared as described previously (Tranum-Jensen *et al.*, 1978). To 1 vol. of erythrocytes at 37°C was added 1.5 vol. of rabbit serum. Lysis was complete within 2 min. Membranes were pelleted, washed, and the packed membranes were quantitatively solubilized with 250 mM-deoxycholate by addition of the solid detergent and agitation at room temperature for 15–20 min (Biesecker *et al.*, 1979). The solubilized material was applied in 1 ml portions to linear 10–50% (w/v) sucrose density gradients [12.4 ml total volume, prepared in 5 mM-Tris/25 mM-NaCl (pH 8.2) / 7.5 mM - NaN_3 / 6.25 mM - deoxycholate]. Centrifugation was at 150000 *g* for 16h at 4°C (Beckmann ultracentrifuge; rotor SW 41Ti). From the bottom of the tubes 20 equal fractions were collected manually. The C5b–9(m) complex was recovered in fractions 2–8, corresponding to 40–25S. Human C5b–9(m) used for analytical comparisons was isolated in parallel by identical procedures.

Isolation of rabbit SC5b–9

Rabbit serum was activated with particulate inulin, and isolation of SC5b–9 was essentially as described for human SC5b–9 (Bhakdi & Roth, 1981).

Immunoelectrophoresis

Electroimmunoassays were as described previously (Tranum-Jensen *et al.*, 1978). Pig antibodies to rabbit serum protein (code 210 SX) were obtained from Dakopatts Immunoglobulins, Copenhagen, Denmark, and used for monitoring the protein separations.

SDS/polyacrylamide-gel electrophoresis

Unidirectional and two-dimensional SDS/polyacrylamide-gel electrophoresis, and polypeptide mapping with *Staphylococcus aureus* proteinase using two-dimensional SDS/polyacrylamide-gel electrophoresis were performed as described previously (Bhakdi & Tranum-Jensen, 1982*b*; Bordier & Crettol-Järvinen, 1979).

Electron microscopy

Sucrose-density-gradient fractions were dialysed against buffer containing 6.25 mM-deoxycholate to remove sucrose before negative staining with sodium silicotungstate at pH 7.3 (Tranum-Jensen *et al.*, 1978). In addition, intact sheep erythrocyte membranes lysed with human or rabbit serum were examined with negative staining before and after proteolytic treatment.

Results

Rabbit C5b–9(m)

Sheep erythrocyte membranes lysed with rabbit complement were solubilized with deoxycholate. Centrifugation through linear sucrose density gradients containing 6.25 mM-deoxycholate separated one high-molecular-weight protein from the bulk of contaminating serum protein. The immunoprecipitation pattern obtained by fused rocket immunoelectrophoresis was very similar to that obtained when erythrocytes were lysed with human complement and the immunoplate developed with an antiserum to human serum proteins (Bhakdi & Tranum-Jensen, 1982*a*). The protein was absent in detergent extracts of control membranes treated with heat-inactivated serum. It sedimented as a broad peak (25–40 S) as previously described for human C5b–9(m) (Bhakdi & Tranum-Jensen, 1981, 1982*a*) and was subsequently identified as rabbit C5b–9(m) on the basis of its ultrastructure and subunit composition. Initial experiments ascertained that native erythrocyte membrane proteins did not sediment to regions corresponding to >25 S, and contamination with membrane constituents was therefore considered negligible.

In the electron microscope, the classical complement lesions, seen in negative stainings of membranes lysed with rabbit serum, appeared morphologically indistinguishable from the lesions produced by human serum in parallel experiments (results not shown). Examination of 26–29 S fractions from the sucrose-density-gradient separations of complement-treated membrane extracts consistently showed the appearance of the typical hollow-cylinder structure previously described for human C5b–9(m) (Fig. 1). Some of the cylinders, seen as rings in axial projection, exhibited a defect of closure. Similar 'open' rings may be found in preparations of human C5b–9(m) as well. Whether they occur *in situ* on freshly lysed membranes or whether they arise during the isolation procedure requires investigation.

As with detergent-solubilized human C5b–9(m), earlier fractions from the gradient corresponding to ⩾33 S always contained aggregates of these unit structures (Fig. 1*c*). On SDS/polyacrylamide-gel electrophoresis, all fractions containing the depicted protein (fractions 2–8 from the gradient) exhibited an identical and consistent polypeptide banding pattern (Fig. 2, gel *b*). Human C5b–9(m) prepared

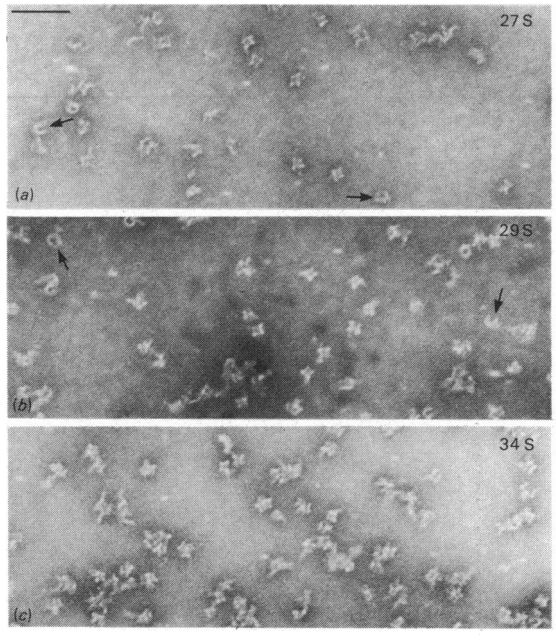
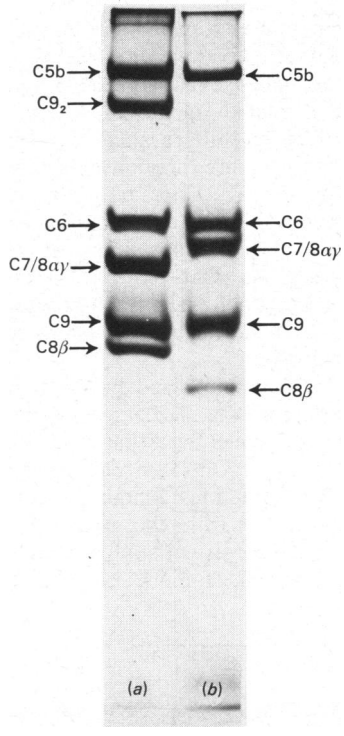


Fig. 1. *Fractions from the sucrose-density-gradient separation of extracts of membranes lysed with rabbit serum*

Negative staining was with sodium silicotungstate. Most of the material present in fractions in the 26–29 S region possess the hollow cylindrical structure characteristic of human C5b–9(m) (*a* and *b*). Arrows point to axial views of cylinders exhibiting an apparent defect of closure. Earlier fractions from the gradient contain similar structures in aggregated form (*c*). Scale bar indicates 100 nm.



in parallel is shown in Fig. 2, gel (*a*). It is apparent that the electrophoretograms show basic similarities. The designations of the polypeptide bands of rabbit C5b–9(m) (Fig. 2), at first tentative, were borne out by subsequent two-dimensional gel-electrophoretic analyses.

Human and rabbit C5b–9(m) were electrophoresed in SDS under non-reducing conditions in the first dimension, followed by second-dimension electrophoresis under reducing conditions. Figs. 3(*a*) and 3(*b*) show the two-dimensional patterns obtained, and the similarities between the two are evident. The two-dimensional pattern of human C5b–9(m) was exactly as previously described by Ware & Kolb (1981), apart from the displacement of C7 and C8αγ as reported previously (Bhakdi &

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of human (a) and rabbit C5b–9(m) (b)*
Designation of the human complement components is as described by Ware & Kolb (1981). The designation of the rabbit components was based on two-dimensional SDS/polyacrylamide-gel electrophoresis analyses (see Figs. 3 and 4). Note the absence of the C9 dimer band in rabbit C5b–9(m).

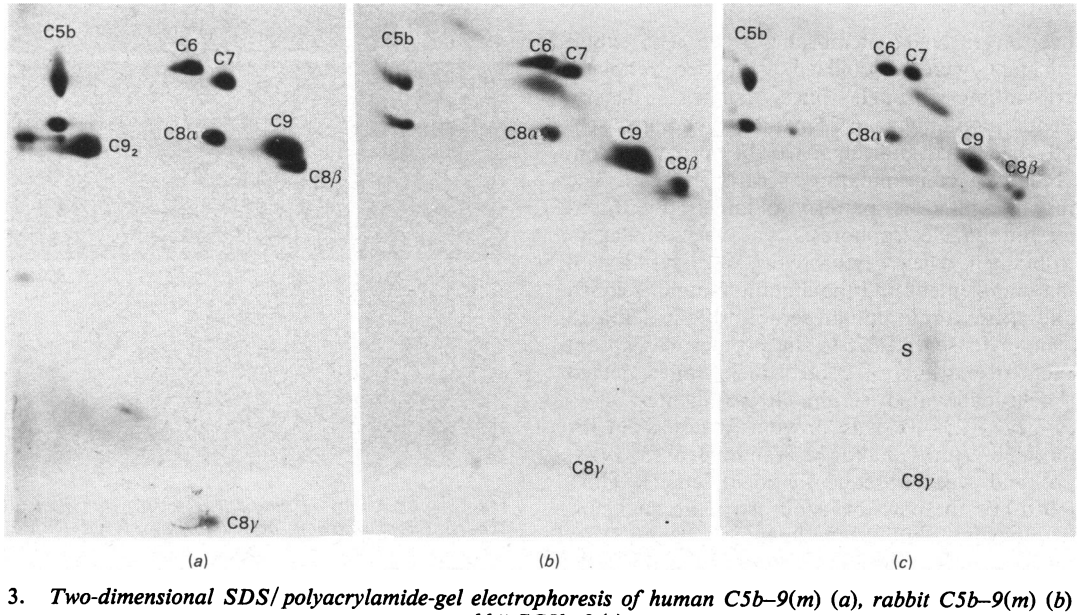


Fig. 3. *Two-dimensional SDS/polyacrylamide-gel electrophoresis of human C5b–9(m) (a), rabbit C5b–9(m) (b) and rabbit SC5b–9 (c)*
First-dimension electrophoresis (left to right) was performed under non-reducing conditions. Second-dimension electrophoresis (top to bottom); proteins were electrophoresed through a strip of agarose containing 200mM-dithiothreitol to effect cleavage of disulphide bonds. Note the behaviour of rabbit C5b, C6, C7 and C8αγ, analogous to that of human components, the cleavage of the S-protein, and the absence of C9-dimer in rabbit C5b–9(m), in contrast with human C5b–9(m).

Tranum-Jensen, 1982*b*). It can be seen that C5b, the C9 dimer and C8 were cleaved by dithiothreitol to yield their respective subunits. Similarly, the protein bands corresponding to rabbit C5b and C8$\alpha\gamma$ split to yield analogous subunits (compare Figs. 3*a* and 3*b*). Conspicuously, no protein band found in the rabbit complex ever split to yield a subunit corresponding to monomeric C9.

In order to obtain additional evidence for homology between the non-disulphide-bonded components of human and rabbit C5b–9(m), polypeptide maps were prepared by using two-dimensional SDS/polyacrylamide-gel electrophoresis. The proteins were first separated under non-reducing conditions in SDS. The SDS-denatured and separated proteins were transferred in the polyacrylamide-gel strips horizontally on to polyacrylamide-gel slabs. Cleavage of the individual polypeptides was then performed with *S. aureus* proteinase during the second-dimension electrophoresis. The polypeptide maps obtained are depicted in Fig. 4. Discrete polypeptides were generated from each human complement subunit with the proteinase, and these were as previously reported for SC5b–9 (Bhakdi & Tranum-Jensen, 1982*b*). A map of the rabbit terminal complex is shown in Fig. 4(*b*). The unidirectional gels shown in Fig. 2 may be placed horizontally on top of the gel slabs for reference and comparison. Similarities between the polypeptide maps generated from human C5b, C6, and C7 or C8$\alpha\gamma$ with corresponding subunits of rabbit C5b–9(m) are apparent (arrows, Fig. 4). The C7 and C8$\alpha\gamma$ maps were not well resolved, and no attempt was made to improve the patterns, because the two-dimensional electrophoreses of Fig. 3 had already provided good evidence for the homologies of these proteins. The region of C9 and C8β was of primary interest. SDS-denatured human C9 was cleaved to yield four major fragments (Fig. 4*a*). The rabbit protein migrating similarly to human C9 also yielded four major fragments, of which three appeared homologous with the human C9 polypeptides (arrows, Fig. 4). Similarly, two peptide-map products of human C8β appeared to be homologous with fragments generated from the last and smallest rabbit protein subunit. The polypeptide maps thus support the designation of the subunits as given in Fig. 2. In particular, they show that the band representing rabbit C9 is homogeneous and not

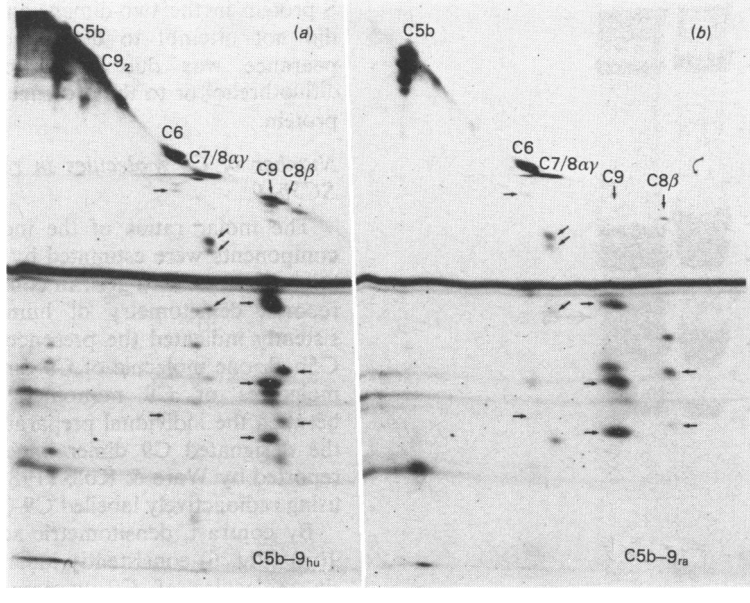


Fig. 4. *Polypeptide mapping by two-dimensional SDS/polyacrylamide-gel electrophoresis of human* (*a*) *and rabbit* (*b*) *C5b–9(m)*

First-dimension electrophoresis (left to right) was in SDS as in Fig. 2. Gel strips were then transferred horizontally on to gel slabs and cleavage of individual subunits was effected by *S. aureus* proteinase during second-dimensional electrophoresis (top to bottom). Note the homologies between human and rabbit C9 and C8β in particular. The C9 dimer in human C5b–9(m) did not map out as monomeric C9, owing probably to alteration of the map products through the prevailing disulphide linkages between the two C9 molecules. Horizontal lines derive from *S. aureus* proteinase.

contaminated by any accidentally co-migrating protein.

Assuming the designation to be correct, three salient points emerge. Firstly, rabbit C5b–9 complement components have approximately the same molecular weight as their human counterparts. Minor deviations pertain to rabbit C8β, which is of somewhat lower molecular weight than human C8β, and rabbit C7/C8$\alpha\gamma$, which both appear slightly larger than human C7/C8$\alpha\gamma$. (The gels of Figs. 3*a* and 3*b* were run independently, and gel *a* was electrophoresed for a slightly longer time than gel *b*. Later experiments have indicated that rabbit and human C8γ do not significantly differ in apparent molecular weight from one another.) Secondly, whereas human C5b–9(m) always contained disulphide-bonded C9 dimers, no such polypeptide band was ever found in rabbit C5b–9(m). Thirdly, instead of the two to four molecules of monomeric C9 present in human C5b–9(m) (Bhakdi *et al.*, 1976; Biesecker *et al.*, 1979; Ware & Kolb, 1981), there were, by densitometry, consistently six molecules of monomeric C9 present for each rabbit C5b–8 monomer (Fig. 6 below). The molar ratio obtained for rabbit C5b–C8 components was reproducibly 1:1:1:1 by the same method.

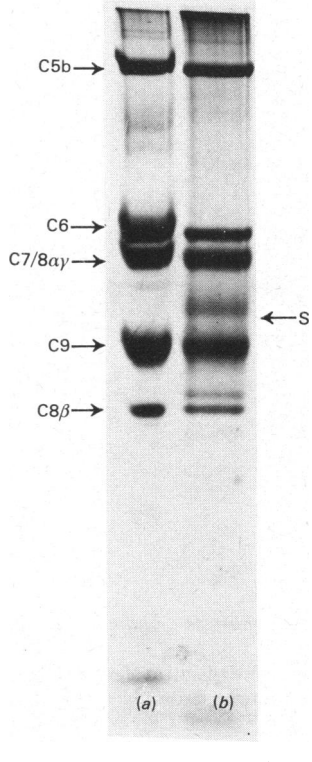


Fig. 5. *SDS/polyacrylamide-gel electrophoresis of rabbit C5b–9(m) (a) and SCb–9 (b)*

Rabbit SC5b–9

When rabbit serum was activated with inulin, the protein purification scheme described previously for human SC5b–9 led to the isolation of a 23 S protein fraction displaying the polypeptide subunit composition shown in Fig. 5 (gel *b*). The major polypeptide bands appeared to match those of rabbit C5b–9(m) (gel *a*). In addition, there was another protein migrating between C7/C8$\alpha\gamma$ and C9, positionally corresponding to human S-protein (Kolb & Müller-Eberhard, 1975; Bhakdi & Tranum-Jensen, 1982*b*). For reasons that are unclear at present, the intensity of this band varied. The rabbit SC5b–9 preparations contained several protein contaminants that were detectable by immunoelectrophoresis and by two-dimensional gel electrophoresis (see Fig. 3*c*). However, the relatedness of the major protein subunits to those of rabbit C5b–9(m) was apparent (compare Figs. 3*b* and 3*c*). The protein probably representing rabbit S-protein was partially cleaved by dithiothreitol to one major subunit of mol.wt. 42000, a behaviour that was analogous to that of human S-protein (Bhakdi & Tranum-Jensen, 1982*b*). The intensities of the protein spots occupying the original positions of rabbit C6/C7 and S-protein in the two-dimensional plates varied. We did not attempt to determine whether their appearance was due to incomplete cleavage by dithiothreitol or to the presence of truly uncleavable protein.

Number of C9 molecules in rabbit C5b–9(m) and SC5b–9

The molar ratios of the individual complement components were estimated by densitometric evaluation of the stained gels. In confirmation of previous reports, densitometry of human C5b–9(m) consistently indicated the presence of one molecule of C5b–8, one molecule of C9 dimer, and two to four molecules of C9 monomer, the latter varying between the individual preparations. The identity of the designated C9 dimer band (Fig. 2) with that reported by Ware & Kolb (1981) was confirmed by using radioactively labelled C9 (results not shown).

By contrast, densitometric scans of rabbit C5b–9(m) (Fig. 6) consistently indicated the presence of six molecules of C9 monomer per molecule of C5b–8. Rabbit SC5b–9, however, contained only two to three molecules of C9 for each molecule of C5b–8. This ratio was repeatedly obtained through comparison and evaluation of the C9/C6 bands. From the results shown in Fig. 3, we believe that the impurities contained in SC5b–9 preparations should not have been able to affect significantly the result of this approximation.

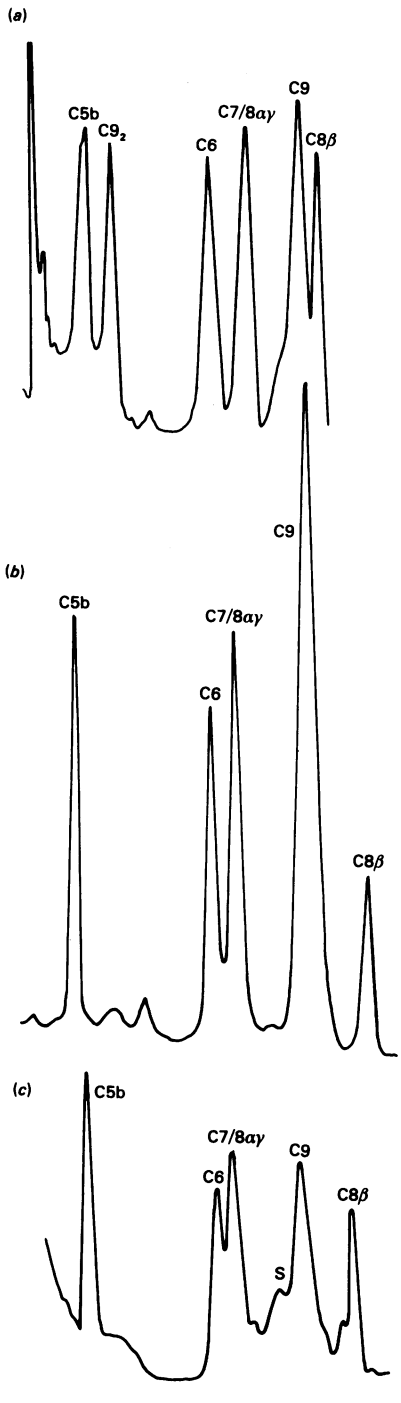


Fig. 6. *Densitometric scans of human C5b–9(m) (a), rabbit C5b–9 (b) and rabbit SC5b–9 (c)*
Note the absence of C9 dimer in rabbit C5b–9(m), the higher number of C9 monomers in rabbit C5b–9(m) compared with human C5b–9(m) (six instead of three to four), and the relatively low amount of C9 in rabbit SC5b–9 (two to three molecules per complex).

Molecular weight of rabbit C5b–9(m)

The hydrodynamic properties of rabbit C5b–9(m) were equivalent to those previously reported for human C5b–9(m). As discussed by Bhakdi & Tranum-Jensen (1981), a sedimentation coefficient of 29S would be comparable with a molecular weight of approx. 1.4×10^6 for the protein–detergent complex. Detergent binding was not measured for rabbit C5b–9(m), but, assuming it to be similar to that of human C5b–9(m), a protein of $(1–1.1) \times 10^6$ would be obtained.

Discussion

After lysis of antibody-coated sheep erythrocytes with rabbit serum, we isolated a protein from deoxycholate/detergent extracts of the target membranes that, by virtue of its ultrastructural and biochemical properties, must surely represent the rabbit C5b–9(m) complex. The protein was not present in detergent extracts of control membranes treated with inactivated serum. It exhibited a hollow cylindrical structure similar to that of human C5b–9(m). Gel-electrophoretic analyses employing two-dimensional techniques indicated the presence of protein subunits homologous with human C5b–C9. These findings encouraged a comparison between the biochemical properties of human and rabbit C5b–9(m).

Rabbit C5b–9(m) contains C5b–C8 in a molar ratio of 1:1:1:1, as does human C5b–9(m). By densitometry, however, there are six molecules of monomeric rabbit C9 per molecule of C5b–8. By contrast, human C5b–9(m) contains one molecule of disulphide-linked C9 dimer and less monomeric C9 per complex. Common to both rabbit and human C5b–9(m), therefore, is the presence of multiple (probably six) C9 molecules in each monomer. The present estimate of six molecules C9 per complex shares with all other previous calculations the uncertainty of being based on densitometric quantification. For human C5b–9(m), however, this value does agree very well with the theoretical value obtained from careful binding studies (Kolb *et al.*, 1972). By extrapolation, we thus regard the values obtained for rabbit C5b–9 in the present study with some confidence.

Fluid-phase rabbit SC5b–9 consistently contained fewer C9 molecules per C5b–8 monomer. This was in accord with the available data on human C5b–9 and supports the notion that fluid-phase complement complexes do contain fewer C9 molecules than do their fully assembled membrane counterparts (Ware & Kolb, 1981).

The molecular weight of SC5b–9 is approx. 1.05×10^6 (Kolb & Müller-Eberhard, 1975). The theoretical molecular weight of monomeric C5b–9(m) would be of the same order of magnitude, since

the two to three molecules of S-protein per SC5b–9 complex are replaced molecular-weight-wise by the additional C9 molecules. C5b–9(m) is an amphiphilic macromolecule binding large amounts of detergent (Bhakdi & Tranum-Jensen, 1981), and the seemingly high sedimentation coefficient of 29 S thus still indicates a monomer rather than a dimer structure of the complex. Indeed, a C5b–9 dimer as proposed by Biesecker *et al.* (1979) would have to possess the structural formula $(C5b\text{–}8)_2C9_{12}$. The protein molecular weight of such a dimer would be $(2\text{–}2.2) \times 10^6$, and that of the protein–detergent complex would be significantly higher. Thus even a molecular weight of 1.7×10^6, as suggested by Biesecker *et al.* (1979), who assumed a sedimentation coefficient of 33.5 S, is hardly compatible with a dimer nature of the complex. In this connection it is noteworthy that Ware *et al.* (1981) have also found human C5b–9(m) to form a major sedimenting peak of 29 S in deoxycholate-containing sucrose density gradients. These authors used a value of 0.844 as partial specific volume and tentatively calculated a molecular weight of 2.94×10^6 for the complex (Ware *et al.*, 1981). This high value for the partial specific volume, taken from another study (Podack *et al.*, 1979), was, however, not correct, because it pertained to protein–lipid and not to protein–detergent complexes (Podack *et al.*, 1979). Clearly, the protein (partial specific volume: 0.72)–deoxycholate (partial specific volume: 0.778; Tanford & Reynolds, 1976) complexes cannot have a composite partial specific volume exceeding 0.73–0.74 (assuming detergent binding of 20–25% of protein weight; Bhakdi & Tranum-Jensen, 1981; Tanford & Reynolds, 1976). Therefore we regard the sedimentation coefficient of 29 S found by Ware *et al.* (1981) to be in excellent agreement with our data, and with our proposal that C5b–9(m) is a monomer.

The higher number of C9 molecules in the membrane C5b–9(m) complex compared with its fluid-phase counterpart satisfactorily accounts for the relatively high molecular-weight estimate of 10^6 previously suggested by this laboratory. The significance of C9 dimer formation is as yet unknown, but Ware & Kolb (1981) have considered the possibility that it may be connected with expression of membrane-damaging C5b–9(m) function. Rabbit C5b–9(m), however, contains no C9 dimers. Therefore dimer formation through disulphide bridging plays a role neither in formation of the C5b–9(m) cylindrical molecular structure nor in the expression of cytolytic complement function. Additionally, dimer formation can also not provide the biochemical basis for formation of supramolecular C5b–9(m) oligomers in the rabbit system, as proposed by Ware & Kolb (1981) for human C5b–9(m). These investigators have advanced the notion that formation of such oligomers may be responsible for the observed heterogeneity in functional pore size of the complement lesions (Sims & Lauf, 1978; Boyle & Borsos, 1979; Boyle *et al.*, 1979). Our present data do not basically exclude this possibility, but the necessity of obtaining compelling experimental data is obvious, and the use of rabbit complement may provide a useful tool in this respect. Future analyses of C5b–9(m) from other animal species should eventually disclose whether C9 dimer formation is the exception or the rule during complex assembly on the membrane.

In conclusion, these data support the concept initially proposed by Kolb *et al.* (1972) that the membrane-damaging C5b–9 complex has the structural formula $(C5b\text{–}8)_1C9_6$. This concept is difficult to reconcile with the most recent claim of Podack *et al.* (1982) that each ring lesion is composed of twelve C9 molecules, and that cylindrical unit lesions seen in the electron microscope, although apparently uniform in structure, may have the biochemical formula $(C5b\text{–}8)_1C9_{12}$ (monomer complexes) or $(C5b\text{–}8)_2C9_{12}$ (dimer complexes). Podack *et al.* (1982) have based their model on the finding that isolated human C9 can form annular structures identical with those formed by C5b–9(m) (Tschopp *et al.*, 1982), and preliminary data by these authors have indicated a dodecameric structure of such 'poly-C9' tubules. However, molecular-weight determinations of the poly-C9 structures are subject to the same difficulties as those of C5b–9(m), since the majority of poly-C9 cylinders are recovered in aggregated form (Tschopp *et al.*, 1982). The controversies surrounding these issues underline the need of further investigations into the molecular composition of terminal complement complexes after their isolation as ultrastructurally defined entities.

Note added in proof

Yamamoto *et al.* (1982) have furnished evidence that dimerization of C9 in the human complex is catalysed by glutathione that is present in the cell haemolysate, and that C9 dimer formation may not be causally linked to the process of cell binding and membrane damage in the human system. These findings are in basic accordance with our observations on rabbit C5b–9(m).

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