Immunology 1978 **35** 205

The structural events associated with the attachment of complement components to cell membranes in reactive lysis

R. R. DOURMASHKIN *Section of Electron Microscopy, Clinical Research Centre, Harrow*

Received 4 *April* 1977; *accepted for publication* 21 *December* 1977

Summary. Electron microscopic study of the events occurring at the cell membrane during reactive lysis by complement, showed that a foliaceous particle was formed at the $\overline{C5b-7}$ stage, that enlarged to a particle with a variable number of arms at the $\overline{C5b-8}$ stage. Up to this point, no typical complement lesions were found. At the $\overline{C5b-9}$ stage, the particles were completely converted to typical complement lesions, i.e. hollow cylinders projecting from the cell membrane and partly penetrating it. $\overline{C5b-9}$ complexes assembled in the fluid phase did not show the typical structure of the lesions, but were amorphous masses of fibres.

INTRODUCTION

The number of lesions observed by electron microscopy in cell membranes after complement lysis was shown by Borsos, Dourmashkin & Humphrey (1964) to correspond to the predicted number of sites of damage. Later observations showed that clusters of lesions occurred when conditions in which the amplification steps of complement at C4 and C3 generated additional sites of damage (Frank, Dourmashkin & Humphrey, 1970). These and other findings summarized by Humphrey & Dourmashkin (1969) suggested that the lesions observed by electron microscopy corresponded in a one-to-one ratio to the sites of damage caused by complement, and were channels whereby cationic equilibrium occurred, leading to osmotic swelling and cell lysis.

Kolb and his co-workers, using purified complement components, showed that C5b, C6, C7, C8 and C9 formed a complex in the aqueous phase; their assembly on the cell membrane caused the functional lesion of complement (Kolb, Haxby, Arroyave & Müller-Eberhard, 1973; Kolb & Müller-Eberhard, 1973 and Kolb & Müller-Eberhard, 1975). The phenomenon of reactive lysis was shown to involve the attachment of the activated terminal components of complement (C5b to C9) to cell membranes (Thompson & Lachmann, 1970 and Lachmann & Thompson, 1970) and was greatly enhanced by the treatment of cells with sulphydryl compounds such as aminoisothiouronium-HBr (AET) that acted on the cell membrane by either oxidizing membrane lipids or splitting disulphide bonds in membrane protein, or both (Kann, Mengel, Meriwether & Ebbert, 1968).

It was of interest to examine membranes damaged by reactive lysis in the electron microscope, and to observe the intermediate complexes of complement at each stage of attachment.

Correspondence: Dr R. R. Dourmashkin, Section of Electron Microscopy, Clinical Research Centre, Watford Road, Harrow HA1 3UJ, Middlesex, England.

0019-2805/78/0800-0205$02.00

MATERIALS AND METHODS

Complement components

Highly purified human $\overline{C5b, 6}$, C7, C8 and C9 were the generous gift of Drs W. Kolb and H. J. Müller-Eberhard.

Buffers

$\frac{1}{2}$VBS^{-}: Isotonic Veronal-buffered saline was diluted with an equal amount of distilled water and adjusted to pH 7·4.

Cell membranes

Human erythrocytes were treated with AET according to the method of Sirchia & Dacie (1967). After six-fold washing in PBS, the cells were lysed in half-strength PBS and the resulting stroma were washed four times in the same buffer. They were fixed overnight by adding 1 ml 10% formaldehyde (prepared from paraformaldehyde) to 10 ml suspension of stroma. They were then washed four times in $\frac{1}{2}$VBS^{-} by centrifugation at 100,000 *g* for 15 min and counted by phase contrast microscopy.

Preparation of terminal component intermediates

50 μl stroma suspension, containing 2×10^7 membranes, was placed in each of five 2 ml ultracentrifuge tubes. In tubes 2–5, 12 μl $\overline{C5b, 6}$ (3 mg/ml) was added, the tubes shaken and incubated 15 min at room temperature. In tubes 3–5, 30 μl C7 (2·6 mg/ml) was added, shaken, and incubated 30 min. Tubes 1–5 were then filled with 2 ml $\frac{1}{2}$VBS^{-} and centrifuged at 100,000 *g* for 15 min. The stroma were resuspended in the residual buffer remaining in the tubes, and 25 μl C8 (2·5 mg/ml) was added to tubes 4–5. They were shaken, and after 5 min 25 μl C9 (0·5 mg/ml) was added to tube 5. This was shaken, and after 5 min all the tubes were diluted with 2 ml $\frac{1}{2}$VBS^{-}, and centrifuged at 100,000 *g* for 15 min. The supernatants were discarded and the stroma were resuspended in a drop of distilled water. 50 μl of $\frac{1}{2}$% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4 was added to all the tubes, which were then stored at 4°. In one duplicate sample of $\overline{C5b-8}$, a drop of 1 M urea was added to the tube in order to crosslink the free aldehyde groups on protein fixed with glutaraldehyde, according to Peterson & Pease (1970).

Electron microscopy

The membranes treated with complement components were mounted on carbon films according to a modification of the technique of Valentine, Shapiro & Stadtman (1968). A finely drawn pipette was used to insert the membrane suspension underneath a carbon film evaporated onto mica. The carbon film was then transferred to distilled water and then to 2% sodium phosphotungstate, pH 6. A grid made adhesive with cellotape was applied to the dry surface of the carbon film, which was then picked up with clean newspaper. The grid was allowed to dry with a minimum of draining and examined in a Philips EM 300.

RESULTS

Erythrocyte membranes not treated with complement (E)

The electron micrographs of the glutaraldehyde-fixed membranes showed a more irregular surface than has been seen in unfixed membranes. The rolled edge of the membranes showed no adherent particles projecting from the surface, nor could any particles be found in the background, that had been eluted from the membranes during staining. No complement lesions as described by Humphrey & Dourmashkin (1969) could be found in this control specimen or in the intermediate steps of reactive lysis, $\overline{C5b, 6}$, $\overline{C5b-7}$ or $\overline{C5b-8}$.

Erythrocyte membranes treated with $\overline{C5b, 6}$ ($E\overline{C5b, 6}$)

No adherent particles were seen on the membrane surface or along the membrane edge. However, many irregularly shaped particles were seen in the background, presumably having been eluted while the preparation was drying. No complement lesions such as those described by Polley, Müller-Eberhard & Feldman (1971) were seen on the membrane surface (Fig. 1).

Erythrocyte membranes treated with $\overline{C5b, 6, 7}$ ($E\overline{C5b-7}$)

Along the edge of the membranes, particles were found that were attached to the membrane by means of a narrow pedicle, from which extended two or three branching arms (Fig. 2). Some particles appeared larger, with more arms and a wider base; on close examination, these proved to consist of two or more particles close together. The particles

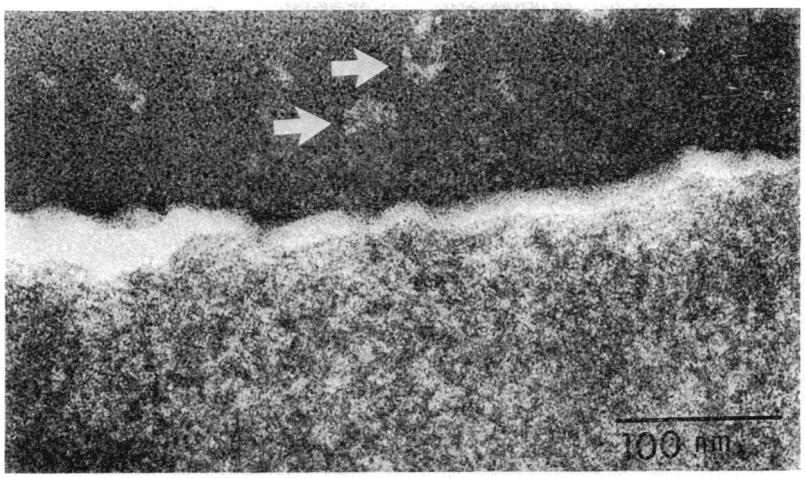

Figure 1. Erythrocyte ghost treated with $C\overline{5b-6}$. A few particles may be seen in the background, eluted from the membrane. The surface of the membrane does not show any complement lesions. (× 240,000).

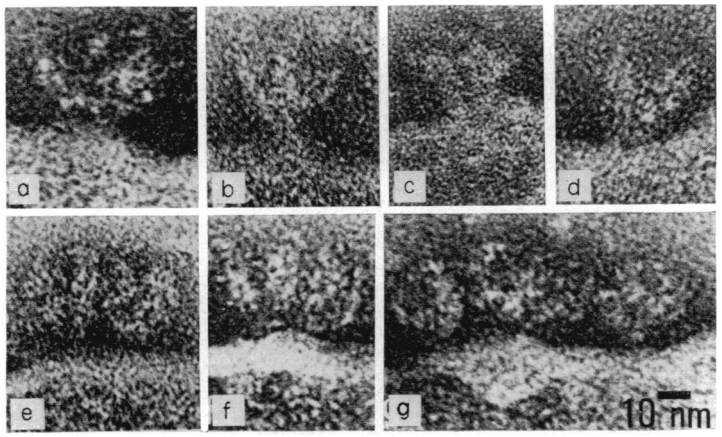

Figure 2. Composite of several micrographs showing erythrocyte ghosts treated with $C\overline{5b-7}$. The particles associated with the attachment of $C\overline{5b-7}$ may be seen projecting vertically from the edge of the membrane. (a) and (b) show particles with three arms; (c) and (d) show particles with two arms; (e) and (f) are particles in groups of two, as they have separate points of attachment; (g) is a group of three or possibly four particles. (× 450,000).

were found along the edge of the membranes in large numbers, up to the point of being crowded close to each other; they were not seen on the membrane surface, probably because of the thickness and irregularity of the membrane. As the observation of these particles corresponded to the addition of C7 to $EC\overline{5b, 6}$, it was concluded that they resulted from the attachment of the stable trimolecular complex $C\overline{5b-7}$ to the cell membrane.

Particles were also found in the background, away from the cell membranes. They were too irregular to interpret.

Erythrocyte membranes treated with $\overline{C5b, 6, 7, 8}$ ($E\overline{C5b-8}$)

Particles were found along the cell membrane edges that were similar to those observed above, mostly crowded together in large numbers (Fig. 3); however, they were considerably bigger than the

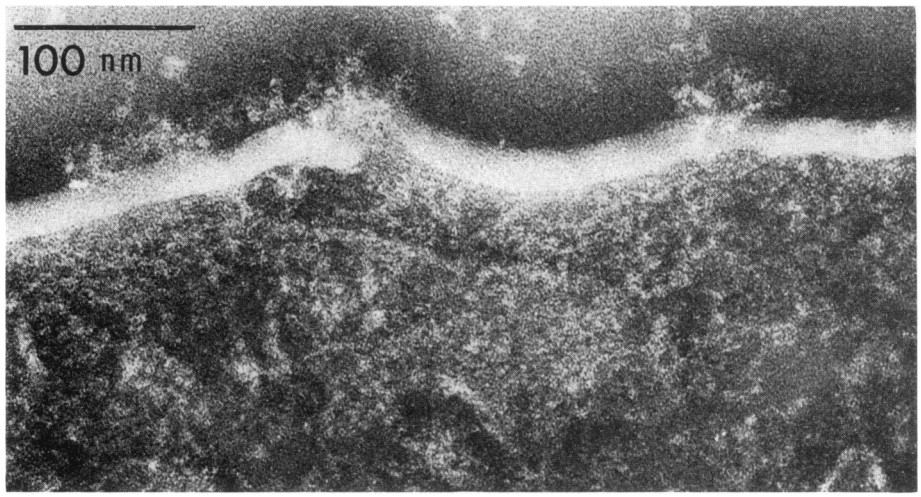

Figure 3. Erythrocyte ghost treated with $C5\overline{6b-8}$. Note the particles projecting from the edge of the membrane. Little is seen on the membrane surface. No complement lesions are found. (× 240,000).

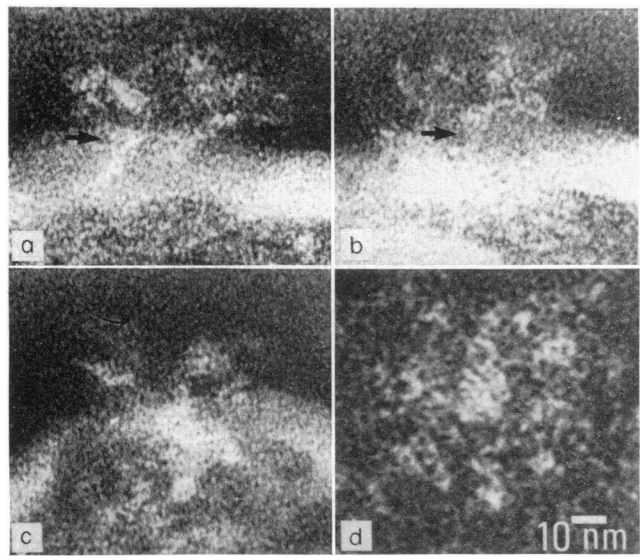

Figure 4. Composite of several micrographs showing erythrocyte ghosts treated with $C5\overline{6b-8}$. (a) and (b) are $C5\overline{6b-8}$ particles seen projecting from the membrane surface, as a lateral view, attached to the membrane surface by a pedicle (→); (c) shows a particle that appears to be viewed vertically, with five arms; (d) is a particle seen on the membrane surface, with approximately six arms, and a central pedicle. (× 450,000).

$C5\overline{6b-7}$ particles. They were attached by means of a pedicle to the membrane surface (Fig. 4a and b) and showed a complicated, possibly variable structure, the details of which were not clear. Like the $C5\overline{6b-7}$ particles, they also showed a foliaceous, branching structure. In those particles that were attached some distance from the folded edge of the membrane, and which were oriented so that the branching arms radiated from the central pedicle (Fig. 4b and c), up to six distinct branches could be

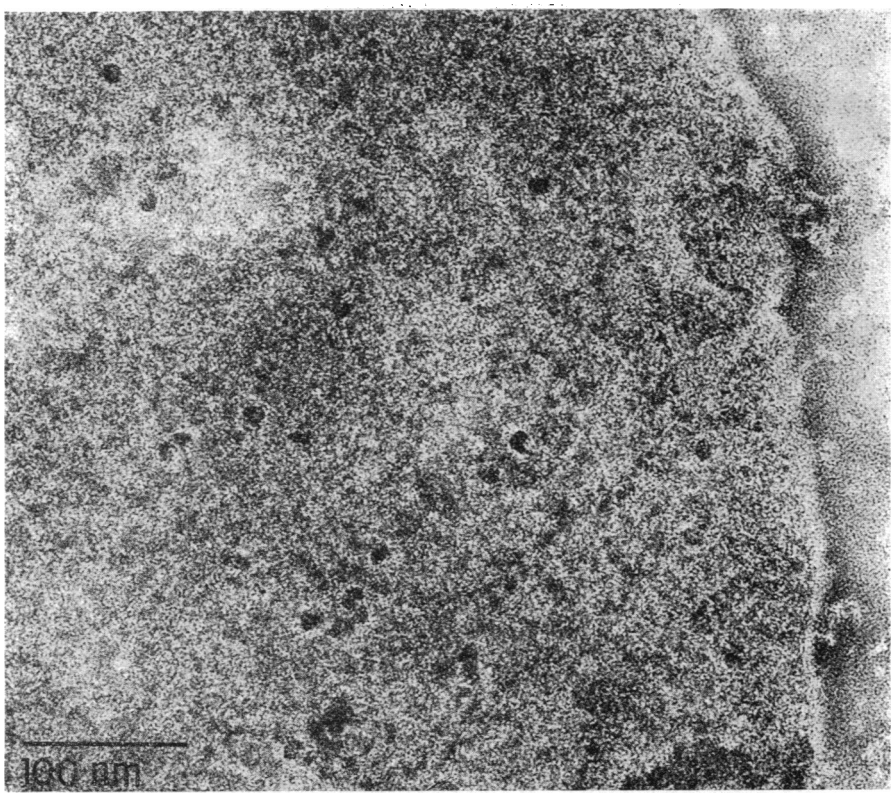

Figure 5. Erythrocyte ghost treated with $C\overline{5b-9}$. A number of typical complement lesions may be seen on the surface, and also projecting from the membrane. (× 240,000).

identified. Irregular particles were also found in the background, although fewer appeared in those membranes treated with both glutaraldehyde and urea. The particles attached to cell membranes also appeared clearer after the urea treatment.

Erythrocyte membranes treated with $C\overline{5b, 6, 7, 8, 9}$ ($EC\overline{5b-9}$)

In this sample, complement lesions were seen on the surface of the membranes (Figs 5 and 6). They were present in large numbers and in clusters over all the membranes examined, whereas not one had been found on the samples previous to the addition of C9. Along the edge of the membranes, the lesions were found to project from the level of the membrane surface, as has been noted previously (Rosse, Dourmashkin & Humphrey, 1966). Viewed in this way in two dimensions, the structure of the lesion was observed to consist of a hollow cylinder with a wide rim at the top. This rim, viewed from above, corresponded to the clear ring around the dark centre. How much of the lesion penetrated into the membrane could not be determined from these electronmicrographs. However, some of the micrographs showed (Fig. 6e and g) that the channel in the lesion penetrated at least part of the membrane thickness.

In addition to their cylindrical structure, some lesions showed projections from the rim edge. The background showed a few small particles and some lesions that were eluted from the cell membrane surface.

$C\ 5\overline{b-9}$ complexes assembled in the fluid phase did not show the characteristic morphology of the complement lesions, but were amorphous collections of fibrous material that did not possess a regular structure.

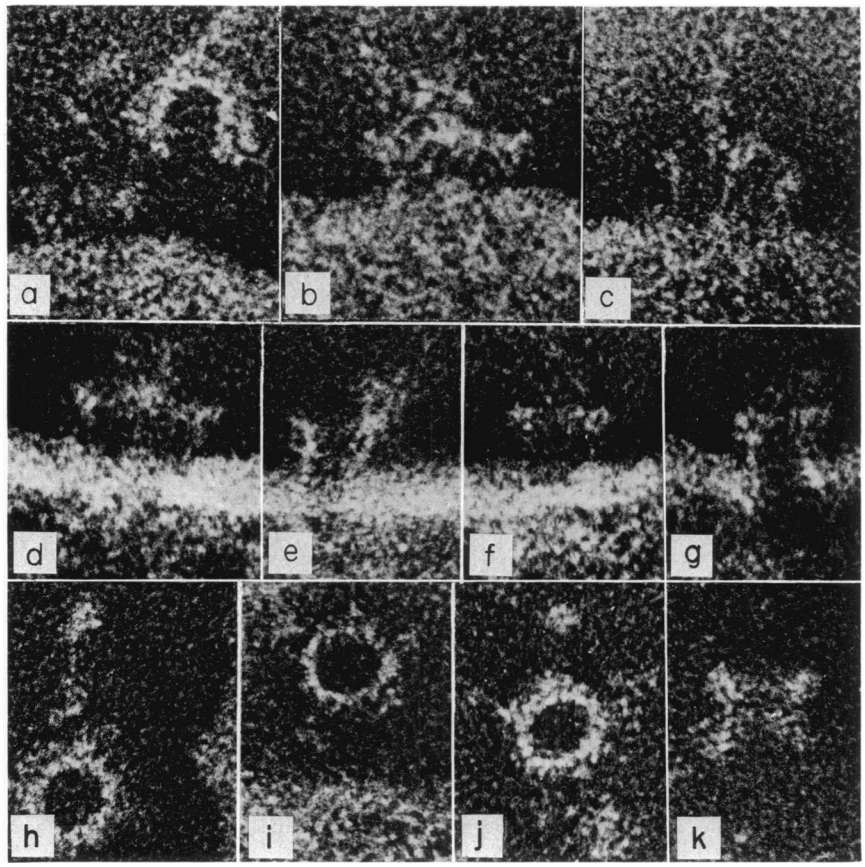

Figure 6. Composite of several micrographs showing erythrocyte ghosts treated with $\overline{C5b-9}$ (a) shows a lesion on the membrane surface, and another that has been eluted from the membrane and is also incomplete; (b), (c) and (d), lesions viewed laterally in groups of two; (e), (f) and (g), single lesions viewed laterally and showing a channel extending into the cell membrane border. (h) to (k) show lesions that had been eluted from the membrane. Small 'tags' may be seen attached to the lesions. (× 600,000).

DISCUSSION

In this study, using the method of reactive lysis, it was found that C9 is required to convert the particles associated with the $\overline{C5b-8}$ complex on the membrane surface into the complement lesions associated with cell lysis. The alteration of the $\overline{C5b-8}$ particles is dramatic. Addition of C9 changes the $\overline{C5b-8}$ particles from a complicated, branching foliaceous structure bound to the membrane surface by a pedicle, to a cylinder with a central channel that penetrates at least part of the membrane lipid layer. The particles associated with the stable trimolecular complex, $\overline{C5b-7}$, is also a foliaceous structure with either two or three arms, similar to the $\overline{C5b-8}$ particles but smaller in size. $\overline{C5b,6}$ is not stable enough to remain attached to the membrane surface after negative staining, even after glutaraldehyde fixation.

Lachmann, Munn & Weissmann (1970) and Lachmann, Bowyer, Nicol, Dawson & Munn (1973) have previously shown that complement lesions could be formed on the surface of liposomes by reactive lysis on the addition of C8 and C9 together to the $\overline{C5b-7}$ complex. However, they were unable to visualize the $\overline{C5b-7}$ particles on liposomes at the $\overline{C5b-7}$ stage, perhaps because post-fixation techniques were not used. Packman, Rosenfeld,

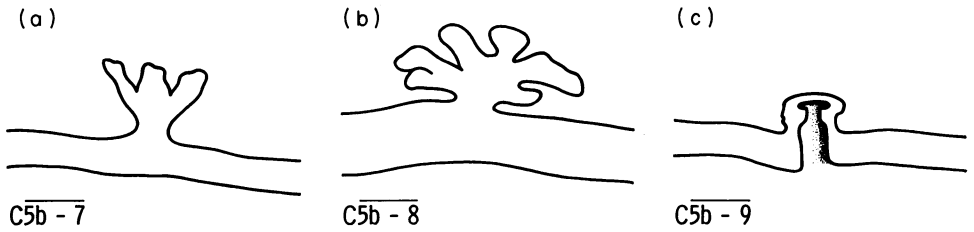

Figure 7. Drawing to illustrate a concept of the attachment of the terminal complement components to cell membranes. (a) $\overline{C5b-7}$; (b) $\overline{C5b-8}$; (c) $\overline{C5b-9}$. It is not known whether the final complement lesion penetrates the entirety of the bimolecular leaflet of the cell membrane.

Weed & Leddy (1976) demonstrated that C9 was required to induce ultrastructural membrane lesions in antibody mediated complement lysis, using human sera deficient in C5, C6, C7 or C8. Using rabbit complement deficient in C6, Humphrey (1972) was unable to demonstrate complement lesions at the C5 stage. These results were at variance with those of Polley, Müller-Eberhard & Feldman (1971), who described complement lesions on the addition of C5 to EAC1423.

Hammer, Nicholson & Mayer (1975) found that 0·3 M NaCl eluted labelled C5 from EAC 1–6 but not from EAC 1–7. This is in agreement with the finding that the attachment of $\overline{C5b-6}$ to the cell membrane is disrupted during the preparation for electron microscopy.

It is possible that the attachment of the terminal components of complement to the cell membrane involves multiple molecules of C7, C8 as well as C9, as opposed to the complex formed in the fluid phase reported by Kolb & Müller-Eberhard (1975). Hammer *et al.* (1975) have shown that both C5b and C7 in EAC 1–7 complexes are partially resistant to removal by trypsin. More recently Hammer, Shin, Abromovitz & Mayer (1977) showed that in EAC 1–8, C8 was highly resistant to proteolytic stripping, and in EAC 1–9, both C8 and C9 were resistant to such treatment. Studies by Shin, Paznekas, Abramovitz & Mayer (1977) and Inoue, Kinoshita, Akiyama, Okada & Amano (1976) showed that phospholipid is released from liposomes and bacterial cell walls as a result of complement damage. These reports support the hypothesis that the terminal components $\overline{C5b-7}$ and $\overline{C5b-8}$ progressively insert themselves into the lipid moiety of the cell membrane. In agreement with these authors, it may be suggested that the $\overline{C5b-7}$ complement complex is partially enveloped by lipid-containing membrane material to produce a particle visible by electron microscopy. This process continues in the $\overline{C5b-8}$ stage, so that the complement components are even more enveloped by the lipid layer.

Up to this point, the particle extends outwards from the bimolecular leaflet in a foliaceous structure. At the $\overline{C5b-9}$ stage, the structure of the particle, containing both complement components and membrane lipoproteins, is altered so that a channel is formed within the bimolecular leaflet to form the complement lesion (Fig. 7). This cannot occur in the absence of membrane lipid or liposomes. The extent to which the bimolecular leaflet of the cell membrane is penetrated is under investigation.

ACKNOWLEDGMENTS

The author wishes to thank Drs W. Kolb, E. Podack and H. J. Müller-Eberhard of the Scripps Clinic, La Jolla, for their kind gift of purified complement components, and their interesting comments, and Dr M. M. Mayer of Johns Hopkins Medical School, for helpful discussion.

REFERENCES

Borsos T., Dourmashkin R.R. & Humphrey J.H. (1964) Lesions in erythrocyte membranes caused by immune haemolysis. *Nature*, **202**, 251.

Frank M.M., Dourmashkin R.R. & Humphrey J.H. (1970) Observations on the mechanism of immune haemolysis: importance of immunoglobulin class and source of complement on the extent of damage. *J. Immunol.* **104**, 1502.

Hammer C.H., Nicholson A. & Mayer M.M. (1975) On the mechanism of cytolysis by complement: evidence on insertion of C5b and C7 subunits of the C5b, 6, 7 complex into phospholipid bilayers of erythrocyte membranes. *Proc. nat. Soc. Acad. Sci., U.S.A.* **72**, 5076.

HAMMER C.H., SHIN M.L., ABRAMOVITZ A.S. & MAYER M.M. (1977) On the mechanism of cell membrane damage by complement; evidence on insertion of polypeptide chains from C8 and C9 into the lipid layer of erythrocytes. *J. Immunol.* **119**, 1.

HUMPHREY J.H. & DOURMASHKIN R.R. (1965) Electron microscopic studies of immune cell lysis. In: *Ciba Foundation Symposium on Complement*, pp. 175–180 (ed. by G. E. W. Wolstenholme and Julie Knight). J. A. Churchill Ltd., London.

HUMPHREY J.H. & DOURMASHKIN R.R. (1969) The lesions in cell membranes caused by complement. *Adv. Imm.* **11**, 75.

HUMPHREY J.H. (1972) The nature of complement-induced lesions in membranes. *Haematologica*, **6**, 319.

INOUE K., KINOSHITA T., AKIYAMA Y., OKADA M. & AMANO T. (1976) Release of phospholipids from bacterial surface structure and liposomes by the action of complement. *J. Immunol.* **116**, 1736.

KANN H.E., MENGEL C.E., MERIWETHER W.D. & EBBERT L. (1968) Production of *in vitro* lytic characteristics of paroxysmal nocturnal hemoglobinuria erythrocytes in normal erythrocytes. *Blood*, **32**, 49.

KOLB W.P., HAXBY J.A., ARROYAVE C.M. & MÜLLER-EBERHARD H.J. (1973) The membrane attack mechanism of complement: reversible interactions among the five native components in free solution. *J. exp. Med.* **138**, 428.

KOLB W.P. & MÜLLER-EBERHARD H.J. (1973) The membrane attack mechanism of complement: verification of a stable C5–9 complex in free solution. *J. exp. Med*, **138**, 438.

KOLB W.P. & MÜLLER-EBERHARD H.J. (1975) The membrane attack mechanism of complement: isolation and subunit composition of the $\overline{C5b\text{–}9}$ complex. *J. exp. Med.* **141**, 724.

LACHMANN P.J. & THOMPSON R.A. (1970) Reactive lysis—the complement mediated lysis of unsensitised cells. II. The identification of activated reactor as $\overline{C56}$ and the participation of C8 and C9. *J. exp. Med.* **131**, 643.

LACHMANN P.J., MUNN E.A. & WEISSMANN G. (1970) Complement-mediated lysis of liposomes produced by the reactive lysis procedure. *Immunology*, **19**, 983.

LACHMANN P.J., BOWYER D.E., NICOL P., DAWSON R.M.C. & MUNN E.A. (1973) Studies on the terminal stages of complement lysis. *Immunology*, **24**, 135.

PACKMAN C.H., ROSENFELD S.I., WEED R.I. & LEDDY J.P. (1976) Complement-induced ultrastructural membrane lesions: requirement for terminal components. *J. Immunol.* **117**, 1883.

PETERSON R.G. & PEASE D.C. (1970) Polymerizable glutaraldehyde-urea mixtures as water-soluble embedding media. *Proc. 28th Ann. Conf. E.M.S.A.*, p. 334.

POLLEY M.J., MÜLLER-EBERHARD H.J. & FELDMAN J.D. (1971) Production of ultrastructural membrane lesions by the fifth component of complement. *J. exp. Med.* **133**, 53.

ROSSE W.F., DOURMASHKIN R. & HUMPHREY J.H. (1966) Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. III. The membrane defects caused by complement lysis. *J. exp. Med.* **123**, 969.

SHIN M.L., PAZNEKAS W.A., ABRAMOVITZ A.S. & MAYER M.M. (1977) On the mechanism of membrane damage by C: exposure of hydrophobic sites on activated C proteins. *J. Immunol.* **119**, 1358.

SIRCHIA G. & DACIE J.V. (1967) Immune lysis of AET-treated normal red cells (PNH-like cells). *Nature*, **215**, 747.

THOMPSON R.A. & LACHMANN P.J. (1970) Reactive lysis—the complement mediated lysis of unsensitised cells. I. The characterisation of indicator factor and its identification as C7. *J. exp. Med.* **131**, 629.

VALENTINE R.C., SHAPIRO B.M. & STADTMAN E.R. (1968) Regulation of glutamine synthetase. II. Electron Microscopy of the enzyme from *Escherichia coli*. *Biochemistry*, **7**, 2143.