Biochimica et Biophysica Acta, 356 (1974) 300–308

BBA 76702

COMPLEMENT-INDUCED CHANGES IN THE CORE STRUCTURE OF SHEEP ERYTHROCYTE MEMBRANES: A STUDY BY FREEZE-ETCH ELECTRON MICROSCOPY

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(Received March 21st, 1974)

SUMMARY

1. Freeze-etch electron microscopy of sheep erythrocyte membranes treated with antibody plus complement reveals increased roughness of the outer-membrane surface but normal morphology and distribution of the membrane intercalar particles. There is a slight decrease in the number of tangential fractures. Occasional ring-shaped particle aggregates occur on external membrane surfaces.

2. Treatment of sheep erythrocyte membranes with lytic doses of lysolecithin or melittin reduces the number of tangential fractures but also does not affect the morphology or distribution of intercalar particles.

3. However, membranes treated with antibody plus complement, lysolecithin or melittin all exhibit a characteristic change after subsequent proteolytic digestion, i.e. extreme aggregation of intercalar particles. Ghosts produced by hypotonic hemolysis, as well as membranes treated with antibody plus inactive complement, do not show any change in particle distribution following protease treatment. Also, membranes treated with antibody plus C6-deficient rabbit serum show no particle aggregation upon protease treatment.

4. The ring-shaped particle aggregates, occasionally seen on the outer-membrane surface following complement action, do not disappear after protease treatment, but become clustered in a manner suggesting very close association with the membrane intercalar particles.

5. We conclude that complement produces a subtle alteration in the structure of the erythrocyte membrane core, which can be mimicked by lysolecithin and the bee venom polypeptide melittin, both membrane-lytic agents.

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INTRODUCTION

Complement-mediated cytolysis is often accompanied by changes in membrane micromorphology [1], whose functional and structural significance remains obscure. After negative staining these "lesions" appear as electron-lucent rings, 25–28 nm in diameter, surrounding opaque spots 15–17 nm in diameter [1, 2]. Similar defects accompany membrane lysis induced by saponin [3] and the polyene antibiotic, filipin [4, 5], both of which appear to effect membrane disruption by interaction with cholesterol [6].

The action of filipin on biomembranes can be simulated in cholesterol-containing liposomes and a number of liposome systems have been developed which respond analogously to membranes upon reaction with antibody and complement [7]. Initial data suggested that both filipin and antibody plus complement produce transmembrane pores. However, Knudsen et al. [8] and Kataoka et al. [9], using liposomes of defined composition, including specific antigens, found no negatively staining "lesions" after incubation of the liposomes with specific antibody and complement. Also Polley et al. [10] report that the characteristic "lesions" appear on sheep erythrocyte membranes already after their reaction with C5, the fifth major component of the complement sequence. Their studies also show that there is no simple relationship between the appearance of the micromorphologic "lesions" and the impairment of the cells' permeability barriers. Furthermore, Seeman and associates [11, 12], using freeze-etch electron microscopy, conclude that the lesions observed in sheep erythrocyte membranes after complement-mediated cytolysis do not constitute microscopic trans-membrane channels.

The scarcity of well reproducible morphological alterations has therefore prompted us to undertake further studies which might reveal other, more subtle changes in complement-treated membranes. The use of proteases has indeed allowed us to detect a change which is phenomenologically interesting in connection with the concept of complement lysis. Furthermore, our studies reveal the existence of close interactions between complement-induced globule aggregates and membrane intercalar particles.

MATERIALS AND METHODS

We employ sheep erythrocytes stored in acid citrate dextrose at 4 °C for not more than 21 days. Our sources of normal complement are pooled guinea pig or rabbit sera. Rabbit serum deficient in the C6-component of complement [13] was generously provided by U. and K. Rother (Institut für Serologie und Immunologie, Universität Heidelberg, Germany). E. Habermann (Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany) kindly supplied us with purified melittin. We obtain rabbit anti-sheep erythrocyte antiserum from Behring (Marburg, Germany), trypsin and soy bean trypsin inhibitor from Boehringer (Mannheim, Germany), pronase from Calbiochem. (San Diego, Calif., U.S.A.) and lysolecithin from Roth (Karlsruhe, Germany).

Lysis of erythrocytes

Complement lysis. We wash sheep erythrocytes five times in isotonic saline and

make suspensions of 10^9 cells/ml in isotonic veronal-buffered saline, 0.001 M in Mg^{2+} and 0.00015 M in Ca^{2+}. We add 0.1 ml rabbit anti-sheep erythrocyte serum per 5 ml cell suspension, incubate for 5 min at 37 °C and follow with one washing in veronal-buffered saline. To 5-ml samples of cells resuspended at 10^9 cells/ml we add 2.0 ml undiluted guinea pig serum, normal or C6-deficient rabbit serum, or guinea pig serum previously incubated at 56 °C for 2 h. With normal guinea pig or rabbit complement, lysis is complete within 5 min, whereas no lysis occurs when C6-deficient serum or heat-inactivated complement is used. In the latter cases we wash the cells once and subsequently lyse in 5 mM phosphate buffer, pH 8.0, as in ref. 14.

Alternatively, we lyse cells hypotonically as in ref. 14 and use the membranes as substrates for complement action, suspending approx. 10^9 ghosts/ml in veronal-buffered saline and subsequently treating with antibody and complement.

Lysis with lysolecithin and melittin. To suspensions of 10^9 cells/ml we add 1 vol. of lysolecithin or 1 vol. of melittin in veronal-buffered saline to final concentrations of 25 and 150 μg/ml, respectively. In both cases lysis is complete within 3 min at 37 °C. We obtain control membranes by hypotonic lysis as in ref. 14.

Proteolytic digestion of membranes

We suspend membranes to approx. 10^9 membranes/ml in veronal-buffered saline, add trypsin or pronase to final concentrations of 0.5 or 0.1 mg/ml, respectively, and incubate at 37 °C for 20 min. We terminate trypsinisation by addition of 2-fold concentration of trypsin inhibitor, and centrifuge membranes at $280\,000 \times g \cdot \text{min}$ in the cold (Sorvall centrifuge model RC-2B, rotor type SS 34). After two washings in 5 mM phosphate, we proceed with electron microscopic studies.

Conversely, we also first treat hypotonically lysed membranes with trypsin, terminate proteolysis with trypsin inhibition, wash thrice in phosphate buffer, and finally treat these membranes with antibody plus complement.

EDTA extraction of membranes

We suspend hypotonically lysed membranes in 80 vol. of 0.5 mM EDTA, pH 8.0, for 60 min at 37 °C, centrifuge the membranes at $9 \cdot 10^6 \times g \cdot \text{min}$ in a Spinco Ultracentrifuge (model L2-653, rotor type 50.1), and employ these membranes as targets for complement, lysolecithin and melittin. To preclude disturbances of the complement reaction by EDTA, we dialyse membranes for 7 h at 4 °C against veronal-buffered saline containing Ca^{2+} and Mg^{2+} before treatment with antibody and complement.

We stain membranes directly on carbon-coated grids with 2 % uranylacetate, or prefix the membranes with 1 % glutaraldehyde for 15 min, wash twice in buffer, and then stain with 2 % phosphotungstic acid adjusted to pH 7.0.

Freeze-etching

We freeze pelleted membranes on cardboard discs in Freon 22, cooled by liquid nitrogen. We perform fracturing, etching (1–3 min at −100 °C) and replicating with a Balzers model 360 M device as in ref. 15 and examine replicas with a Siemens Elmiskop Ia.

RESULTS

Comparison of ghosts produced by hypotonic hemolysis, complement-mediated cytolysis, and hemolysis with melittin and lysolecithin

The freeze-etch morphology of erythrocyte ghosts produced by hypotonic lysis is as previously described [16].The etched surfaces appear smooth. The fracture faces reveal the typical $\approx$ 90 Å intercalar particles, typically distributed uniformly; in older cells occasional clusters of 5–10 particles are seen.

Ghosts produced by complement-mediated hemolysis exhibit somewhat rougher-etched surfaces. In a very few cases circular aggregates are also visible (Fig. 1a). These appear to correspond to the "lesions" seen by negative staining (Fig. 1b), are noted by Iles et al. [11], and are diffusely scattered. There are fewer tangential fracture faces, but the distribution of intercalar particles is indistinguishable from that of osmotically induced ghosts.

Ghosts isolated after lysis of sheep erythrocytes with either melittin or lysolecithin at the stated concentrations cannot be distinguished morphologically from standard hypotonically lysed membranes. At high concentrations of melittin or lysolecithin the number of tangential fracture faces decreases.

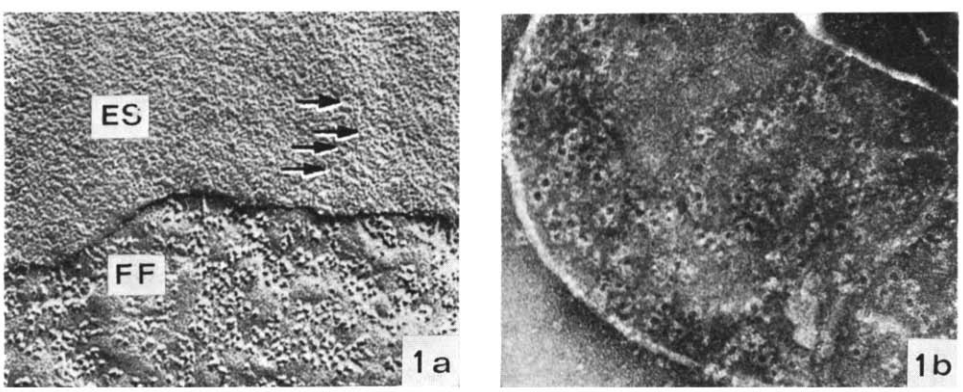


Fig. 1. (a) Sheep erythrocyte ghost produced by complement-mediated lysis after freeze-etching. The somewhat rough-etched surface (ES) bears few circular aggregates (arrows). The fracture face (FF) reveals the randomly distributed membrane particles. Magnification, ×80 000. (b) Sheep erythrocyte ghost produced by complement-mediated hemolysis, negatively stained with uranyl acetate, showing the complement "lesions". Magnification, ×120 000.

Effect of proteases on ghosts produced by hypotonic hemolysis, complement-mediated hemolysis, and melittin and lysolecithin-induced hemolysis

Treatment of hypotonically produced human erythrocyte ghosts with proteases produces aggregation of intercalar particles [16]. This is not observed in the case of sheep erythrocytes (Fig. 2). Indeed, protease-treated sheep membranes cannot be distinguished from untreated, hypotonically produced ghosts, except at pronase levels $>$ 1 mg/ml; then the number of fracture faces decreases and the intercalar particles aggregate slightly and diminish in frequency.

Protease treatment of membranes produced by complement-mediated hemolysis causes extreme aggregation of intercalar particles (Fig. 3). These aggregates

Fig. 2. Hypotonically produced sheep erythrocyte ghost after treatment with pronase. The membrane particles remain unchanged. Magnification, ×80 000.

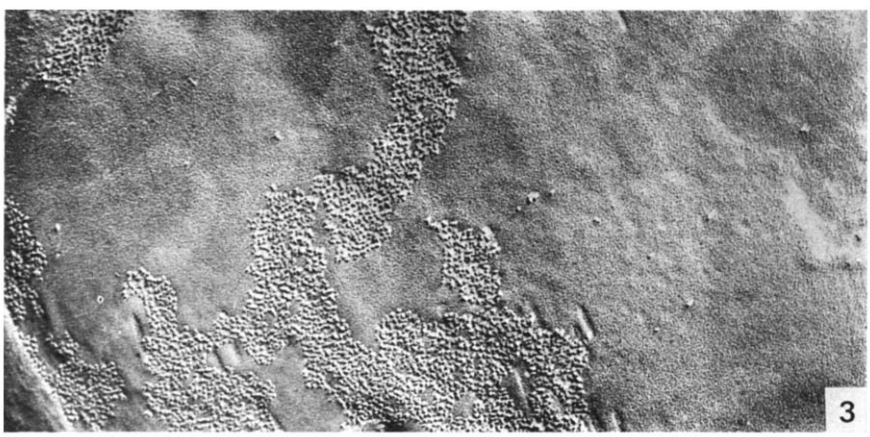


Fig. 3. Protease treatment of sheep membranes produced by complement-mediated lysis causes extreme aggregation of the membrane particles. Magnification, ×80 000.

are separated by extensive smooth areas. The effect is pronounced with both trypsin and pronase, but cannot be mimicked by neuraminidase.

The same is observed after proteolysis of hypotonically produced ghosts treated with antibody plus active complement, but not when heat-inactivated or C6-deficient complement are employed.

Interestingly, many of the replicas obtained after protease treatment still reveal the complement-related ring-shaped globular aggregates on the etched surfaces. These are now better visible, are clustered together and appear associated with aggregated intercalar particles (Fig. 4).

Protease treatment of membranes isolated after melittin or lysolecithin-induced hemolysis also produces massive aggregation of intercalar particles (Figs 5 and 6).

Aggregation of intercalar particles also occurs when proteolytic digestion of

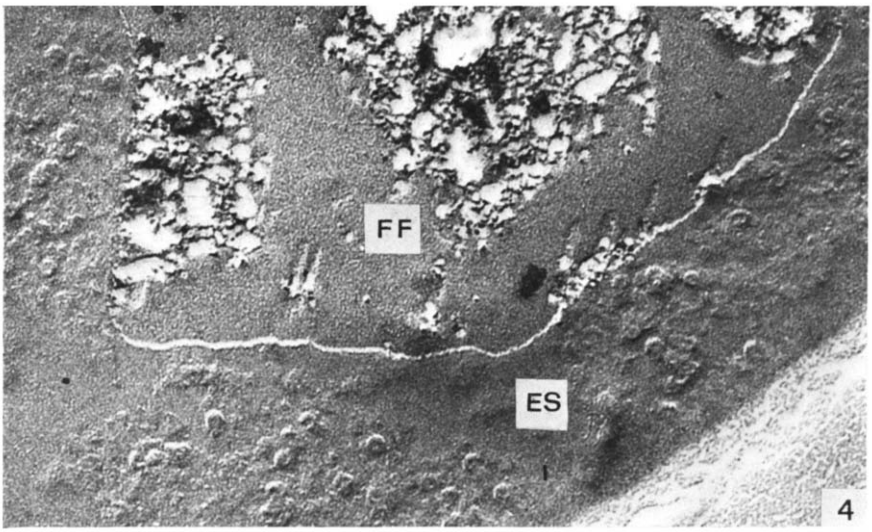


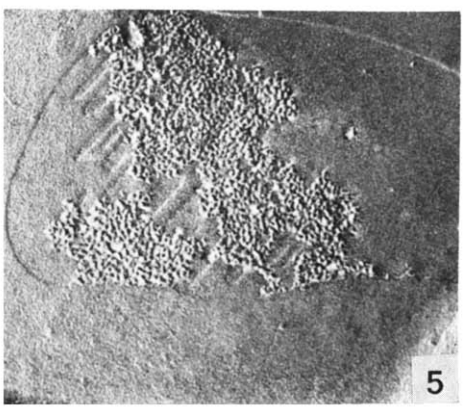
Fig. 4. Same as Fig. 3, note clustering of the complement rings. The clusters lying on the etched surface (ES) are related to the distribution of the underlying membrane particles of the fracture face (FF). Magnification, ×120 000.

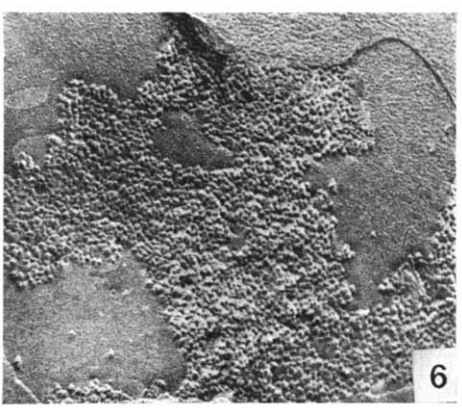
isolated membranes precedes treatment with complement. However, the effect is less dramatic.

Particle aggregation is not demonstrably temperature sensitive. It cannot be induced in hypotonically produced, protease-treated ghosts by chilling or incubating at elevated temperature (37–50 °C). It is also not inducible in complement-, melittin- or lysolecithin-treated membranes by chilling or heating.

EDTA extraction

Membrane extracted with EDTA [17] vesiculate strongly, but exhibit no particle aggregation after treatment with complement, lysolecithin or melittin.





Figs. 5 and 6. Sheep membranes after melittin (Fig. 5) and lysolecithin (Fig. 6) induced hemolysis, treated with pronase. The membrane particles are strongly aggregated. Magnification, ×80 000.

DISCUSSION

Our freeze-etch studies reveal two alterations of sheep erythrocyte membranes following treatment with antibody plus complement. The first "lesion" consists of more or less circular particle aggregates, located on the external membrane surfaces and apparently related to the characteristic circular defects seen by negative staining. These alterations, which are not consistantly present, have been observed by others (cf. refs 5, 7, 10–12) and do not appear related to the complement-induced permeability defect(s) which cause cytolysis [10]. The lesions are not removable by protease treatment and appear to be closely associated with membrane intercalar particles.

We also find that active complement produces a second membrane alteration, not hitherto observed. The exact lesion remains to be defined, but it is clearly manifest as an extensive aggregation of the intercalar particles at the membrane fracture faces upon treating complement-reacted membranes with proteases.

Importantly, no aggregation of intercalar particles occurs after treatment of sheep erythrocyte membranes with trypsin or pronase in the concentrations we use. Membranes treated with inactivated complement or with C6-deficient complement also do not show particle aggregation after protease treatment. However, massive particle clumping appears after protease treatment of membranes exposed to lysolecithin or the lytic polypeptide, melittin, although neither agent can produce the effect alone.

It is well established that freeze-fracture faces reveal apolar membrane cores. Moreover, the evidence indicate that the intercalar particles are protein in nature and that they do not break down upon proteolysis, despite extensive peptide cleavage [16]. In addition, recent experiments (refs 18–21 and Wallach, D. F. H., Speth, V. and Wunderlich, F., unpublished) indicate that particle aggregation, such as here observed, can arise from a lateral segregation of membrane lipid and membrane protein, the smooth areas representing lipid.

Our data indicate that complement "sensitizes" membranes toward such segregation and that this action can be simulated by lysolecithin or melittin, but that the segregation process cannot be realized without cleavage of membrane proteins.

One may discuss two targets for protease action on membranes, which could account for the observed phenomenon. First, cleavage of protein directly associated with the intercalar particles, leading to generation of new, charged groups at the site of action. Second, cleavage of a lattice protein which normally stabilizes intercalar particle distribution. A possible candidate for such a lattice is the erythrocyte protein component "spectrin" which can be eluted by EDTA extraction [17]. However, we find that removal of "spectrin" does not substitute for proteolysis. This does not exclude participation of other proteins in the hypothesized topological stabilization.

At this stage, we cannot distinguish between these possibilities and stress that they concern only mechanisms by which the complement-induced alteration can be visualized.

Because proteolysis alone has no morphological effect on isolated sheep erythrocyte membranes, another process must be involved in particle aggregation following proteolysis of membranes treated with complement, lysolecithin or mellittin. Here we consider two possibilities. First, particle segregation could be brought about by a liquid–solid transition of membrane lipids as a result of complement action.

Such a phenomenon has been observed to occur upon chilling in the nuclear membranes of *Tetrahymena* [18, 21], and lymphocytes (Wallach, D. F. H., Speth, V. and Wunderlich, F., unpublished) as well as in the plasma membrane of *Acholeplasma laidlawii* [18, 20].

However, temperature-sensitive particle segregation cannot be observed in the plasma membranes of lymphocytes (Wallach D. F. H., Speth V. and Wunderlich, F., unpublished), or in erythrocyte membranes, as shown here and in other studies (Speth, V. and Wunderlich, F., unpublished). This may relate to the high cholesterol levels in plasma membranes. Also, we find that the particle aggregation we observe here is quite insensitive to temperature. Moreover, it is simulated by lysolecithin and melittin, both of which tend to disrupt liquid structuring.

We therefore favor a second possibility, namely that complement, lysolecithin and melittin all disorder membrane structure sufficiently to permit the intercalar particles to aggregate by random thermal motion following protease treatment.

Clearly the aggregation phenomenon is not complement specific and can be brought about by at least two distinct "detergents". Of these, lysolecithin has been previously proposed as the final agent in complement-mediated membrane lysis [22], but current evidence does not support this notion. An alternative possibility is that the complement chain ultimately leads to the release of an amphipathic, surface active polypeptide fragment such as melittin, perhaps through proteolytic cleavage of a larger complement component as earlier suggested by Kinsky [7]. This is not excluded by our previous electrophoretic studies on complement action [23], since a small polypeptide would have escaped detection.

We finally point out that the massive derangements reported here merely give a clue to the final mechanism producing complement-mediated membrane damage. Much more minute perturbations, which would not be visible by freeze-etch microscopy, could lead to the permeability defects normally underlying cytolysis.

ACKNOWLEDGEMENTS

We thank Mrs H. Stübig for expert technical assistance.

This investigation was supported by the Alexander von Humboldt Stiftung (S.B.), the Deutsche Forschungsgemeinschaft (V.S.), the Max-Planck-Gesellschaft zur Forderung der Wissenschaften (H.K.), by Grant Ca 12178 of the United States Public Health Service and Award PRA-78 of the American Cancer Society (D.F.H.W.).

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