PRODUCTION OF ULTRASTRUCTURAL MEMBRANE LESIONS BY THE FIFTH COMPONENT OF COMPLEMENT*, ‡

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Subsequent to the action of complement, discrete ultrastructural lesions have been observed on the surface of erythrocytes and bacterial cells. These lesions have a characteristic shape and their diameter varies between 80 and 103 A depending on the source of complement used (1, 2). Employing guinea pig serum as the source of complement, Borsos et al. (3) obtained results which suggested a correlation between the number of lesions observed electron microscopically and the number of functional lesions predicted by the one-hit theory of immune hemolysis (4). However, when human serum was used as the source of complement, many more lesions were observed than had been theoretically predicted (2). The present study was prompted by the resulting uncertainty as to whether the ultrastructural phenomena truly reflect functional membrane lesions. Its purpose is (*a*) to determine the quantitative relationship between ultrastructural lesions and molecules of complement proteins present on cells attacked by complement, and (*b*) to ascertain at which stage during the complement reaction the lesions may first be visualized.

Materials and Methods

Purified Human Complement Components.—Each of the components of human complement were obtained in purified form according to standard procedures: C1 (5), C2 (6), C3 (7, 8), C4 (9), C5 (8), C6 (10), C7,[1] C8 (11), and C9 (12). C2 was used throughout the present study in its oxidized form (^{ox}C2) (13). For some experiments C3 and C5 were labeled with ^{125}I using the chloramine T method (14). In neither case was the hemolytic activity of the component impaired by the labeling procedure and the specific radioactivity for C3 was approximately 30,000 cpm/μg and for C5, 100,000 cpm/μg.

Preparation of Erythrocyte–Antibody–Complement Complexes.—Complexes consisting of

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sheep erythrocytes (E),[2] rabbit antibody to sheep erythrocytes (A), and human complement proteins were prepared as previously described (12, 13).

Determination of the Average Number of Complement Molecules Per Cell.—Specific cellular uptake of C2 (15), C3 (16), and C4 (5) was calculated from input using previously established factors. The input of C4 and C2 was adjusted in order to achieve a 1:1 ratio between cell-bound C4 and C2 (15), and of C3 was adjusted to achieve a 1:100 ratio between cell-bound $\overline{C4,2}$ complexes and C3 molecules. In some experiments, when ^{125}IC3 was used, the number of molecules of C3 bound to the cell was determined directly. Throughout the present work, the number of C5 molecules bound per cell was determined utilizing ^{125}IC5.

Preparation of an Erythrocyte-Complement Complex (EC 5,6,7) in the Absence of Antibody.—The intermediate complex EC5,6,7 was prepared from unsensitized sheep cells by the action of fluid phase $\overline{C4,2,3}$ on C5, C6, and C7, by the method previously described (17).

Removal of Complement Protein from EAC1,4,2,3 and EAC1,4,2,3,5 Complexes.—Trypsin at a final concentration of 0.1% was added to a 2 ml suspension of EAC1,4,2,^{125}IC3 or EAC1,4,2,3,^{125}IC5 cells containing 5×10^8 cells per ml in veronal buffered saline, pH 7.3, containing Ca^{++} and Mg^{++}. Following incubation at 37°C for 15 min, the cells were washed three times in the same buffer and the radioactivity remaining on the cells was determined and compared with that of similar complexes which had been incubated with buffer alone.

Preparation of Stroma for Electron Microscopy.—Sensitized sheep erythrocytes to which had been added all nine components of complement, or whole serum, underwent 100% hemolysis. Intermediate complexes lacking some of the later-acting components were lysed by the addition of distilled water. The number of cells used for preparation of the stroma varied but was never less than 5×10^8. The stroma was washed three times by alternate centrifugation and resuspension in phosphate buffer, pH 6.5, 0.01 M, and the final pellet was suspended in 2% sodium silicotungstate to a dilution of approximately 1:100 and immediately fixed by the addition of a couple of drops of 10% aqueous neutral formalin (18). One drop of this suspension was placed on a 200 mesh grid coated with collodion and carbon.

Calculation of the Number of Lesions Per Cell.—In each experimental situation electron micrographs of at least 10 different membrane fragments were examined. The number of lesions in a measured area of each cell fragment was determined and the mean calculated. From this figure the number of lesions per cell was computed assuming the area of the red cell to be 25.8 sq μ (19).

RESULTS

Quantitative Relationship Between Ultrastructural Lesions and Complement Molecules on the Surface of Lysed Erythrocytes—Sheep erythrocytes were treated with purified human complement proteins so that an average of 1250 $\overline{C4,2}$ complexes became bound per cell. The cell preparation was then reacted with C3 and divided into two portions (A and B) which were incubated with different quantities of C5. After incubation the number of bound C5 molecules per cell was 3640 in A and 71,000 in B. The cells were then lysed by treatment with an excess of C6-C9 and analyzed by electron microscopy. The results, which are listed in Table I, show that the number of ultrastructural lesions corresponded closely to the number of $\overline{C4,2}$ molecules, but not to that of C5 molecules present on these cells.

[2] *Abbreviations used in this paper:* A, rabbit antibody to sheep erythrocytes; E, sheep erythrocytes.

In a similar experiment, $C\overline{4,2}$ and C5 were quantitatively varied (Table II). When the number of C5 molecules was in excess of the number of $C\overline{4,2}$ complexes, the ultrastructural lesions corresponded quantitatively to the $C\overline{4,2}$ complexes present (preparation C). However, when the number of $C\overline{4,2}$ sites was in excess of the number of C5 molecules, the number of lesions corresponded to the latter (D, E, F). It is apparent, therefore, that the ultrastructural lesions induced by complement are related to the number both of $C\overline{4,2}$ and C5 molecules bound to cells. There was no apparent quantitative correlation between

TABLE I

The Relationship Between the Number of Ultrastructural Lesions and the Number of Cell-Bound C5 Molecules When $C\overline{4,2}$ Complexes are Limiting

Cell preparation	No. of $C\overline{4,2}$ complexes per cell*	No. of C5 molecules per cell‡	No. of ultrastructural lesions per cell
A	1250	3,640	1200
B	1250	71,000	1500

* Specific cellular uptake of C2 (15) and C4 (5) was calculated from input using previously established factors.

‡ The number of bound C5 molecules per cell was determined utilizing ^{125}IC5.

TABLE II

The Number of Ultrastructural Lesions Observed When the Numbers of $C\overline{4,2}$ Complexes and C5 Molecules are Varied

Cell preparation	No. of $C\overline{4,2}$ complexes per cell	No. of C5 molecules per cell	No. of ultrastructural lesions per cell
C	1,000	7940	900
D	20,000	3060	1560
E	50,000	2046	1240
F	50,000	7810	3350

cell-bound C3 molecules and ultrastructural lesions, since in each experiment bound C3 molecules were in 100-fold excess of the $C\overline{4,2}$ complexes.

The Complement Reaction Step at which Ultrastructural Lesions Appear—The various intermediate complexes from EA to EAC1,4,2,3,5,6,7,8,9 were prepared sequentially from the same cell suspension and were examined by electron microscopy. As recorded in Table III and documented in Figs. 1 and 2, the ultrastructural lesions first became visible subsequent to the action of C5 and were not increased by the addition of later components. The finding of a small number of lesions in the EAC1-3 complex was due to a slight contamination of C3 with C5. In later experiments utilizing C3 free of C5, no lesions were seen at the EAC1-3 stage of the reaction.

Detection of Lesions on EC5,6,7—In two experiments C5 was bound to un-

sensitized sheep erythrocytes together with C6 and C7 by the action of fluid phase $\overline{C4,2,3}$. The resulting intermediate complex, previously defined as EC5,6,7 (17), could be lysed by C8 and C9. When these cells were examined prior to treatment with C8 and C9, typical ultrastructural lesions were visible.

TABLE III

Analysis of Various Erythrocyte-Complement Complexes for Ultrastructural Lesions

Intermediate complex	Treatment	No. of ultrastructural lesions per cell
EA	None	0
EAC1,4	None	0
EAC1,4,2	None	0
EAC1,4,2,3	None	150*
EAC1,4,2,3,5	None	1705
EAC1,4,2,3,5	C6,7,8,9‡	1590
EAC1,4,2,3,5,6	None	1120
EAC1,4,2,3,5,6	C7,8,9§	1340
EAC1,4,2,3,5,6,7	None	1060
EAC1,4,2,3,5,6,7	C8,9‖	1105
EAC1,4,2,3,5,6,7,8	None	1680
EAC1,4,2,3,5,6,7,8	C9	1509

* C3 was slightly contaminated with C5; when C3 was free of C5 no lesions were seen on EAC1,4,2,3.

‡ Lysis after 90 min at 37°C was less than 100%.

§ 100% lysis occurred in approximately 30 min at 37°C.

‖ 100% lysis occurred in 10 min at 37°C.

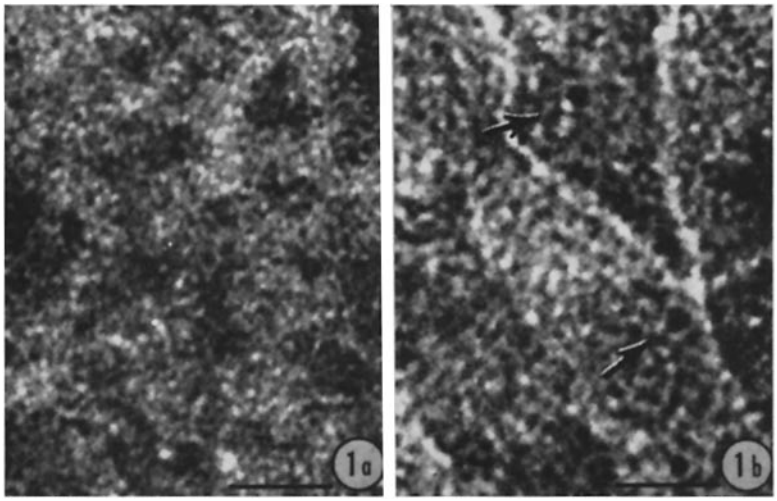

FIG. 1. (*a*) EAC1-3 intermediate complex. (*b*) EAC1-5 intermediate complex. In both cases, stroma prepared by osmotic lysis with H_2O. Solid bar represents 500 A. × 276,000. Representative lesions in (*b*) are indicated by arrows.

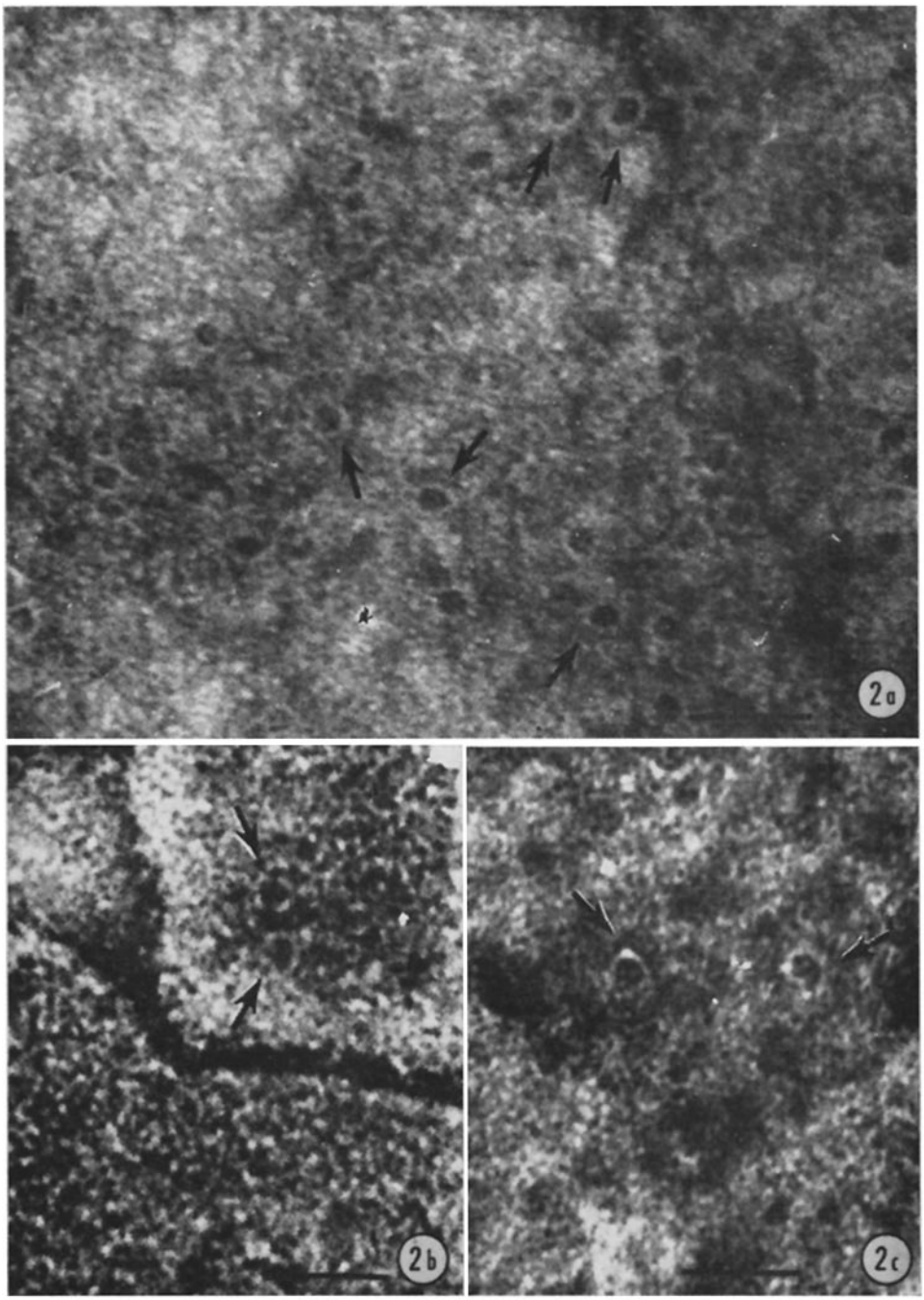

FIG. 2. (*a*) Sheep red cell lysed with whole human complement. (*b*) EAC1-5 intermediate complex. No later components of complement added. Membrane prepared by osmotic lysis with H_2O. (*c*) The same EAC1-5 complex as depicted in 1 *b*, except lysis was induced by the addition of C6-9. Solid bar in each case represents 500 A. × 340,000. Representative lesions are indicated by arrows.

Persistence of Ultrastructural Lesions After Removal of C3 and C5 by Trypsin—Treatment of EAC1,4,2,3 and EAC1,4,2,3,5 with trypsin resulted in removal of approximately 95% of C3 and 100% of C5, as detected by radioactive label introduced into both proteins (Table IV). Stroma prepared from trypsin-treated EAC1,4,2,3,5 was examined by electron microscopy and compared with stroma prepared from the buffer-treated control cells. The number of ultrastructural lesions in both preparations was virtually identical. In the trypsin-treated preparation, the lesions were more clearly visible than in the untreated preparation.

Can C6-Deficient Rabbit Serum Produce Complement-Dependent Ultrastructural Lesions?—Sheep red cells sensitized with rabbit antibody were incubated with normal rabbit serum and with serum from a C6-deficient rabbit. 100% of the cells incubated with the normal rabbit serum lysed within 10 min at 37°C,

TABLE IV

Effect of Removal of Cell-Bound C3 and C5 by Trypsin on Number of Ultrastructural Lesions

Intermediate complex	Treatment	% radioactivity remaining on the cell	No. of ultrastructural lesions per cell
EAC1,4,2,^{125}IC3	Buffer	100	0
EAC1,4,2,^{125}IC3	Trypsin	4.6	0
EAC1,4,2,3,^{125}IC5	Buffer	100	300
EAC1,4,2,3,^{125}IC5	Trypsin	0	320

Number of C3 molecules per cell (untreated), 146,000; number of C5 molecules per cell (untreated), 590. Trypsin treatment: 0.1% solution, 37°C, 15 min.

whereas even after incubation for 90 min, no lysis was obtained with the C6-deficient rabbit serum. The unlysed cells which had been treated with C6 deficient serum were lysed with water and the stroma was compared with that produced by lysis with normal rabbit serum. Ultrastructural lesions were present in both preparations; they were, however, fewer in number in the stroma prepared from cells incubated with C6-deficient rabbit serum than in the control. The difference can probably be explained by the known effect of C6 on C5 uptake. Experiments are under way to test this hypothesis.

DISCUSSION

The first studies on the nature of membrane lesions produced by complement were performed with Krebs ascites tumor cells. Following exposure to antibody and complement, these cells exhibited cytoplasmic swelling, rapid loss of intracellular K^+, amino acids, and ribonucleotides, and less rapid loss of protein and ribonucleic acid (20, 21). The authors explained these changes by assuming the production of functional holes in the cell membrane of sufficient size to permit exchange of small molecules but not of macromolecules. Disturbance of osmotic

regulation was thought to lead to swelling of the cells, "stretching" of the initial lesions, and resulting egress of large molecules (22).

The ultrastructural manifestation of the action of complement on red cells is the appearance of discrete membrane lesions which were originally described as "holes." Similar lesions were produced when lecithin–cholesterol mixtures were treated with saponin (23, 24) and were interpreted by the authors to consist of an inner area of saponin–cholesterol micelles surrounded by a circle of lecithin–cholesterol micelles. Red cells lysed by saponin (24, 25) and by the polyene antibiotic filipin (26) exhibited similar lesions. More recently, studies of the effect of complement on the endotoxic polysaccharide of *Veillonella alcalescens* (27) and on lipid-free and lipid-containing membranes (1) suggested that the lesions first described as holes produced in membranes of red cells subsequent to the action of complement (19, 3) may in fact not be holes, but might well be micelles formed in the lipoprotein layer of the red cell membrane.

In the present study it became apparent that, under the conditions employed, a direct quantitative relationship exists between the number of bound $\overline{C4,2}$ complexes and C5 molecules and the number of ultrastructural lesions. When the number of C5 molecules bound to the cell were in excess of the number of $\overline{C4,2}$ complexes, the latter determined the number of lesions per cell; on the other hand, when the number of $\overline{C4,2}$ complexes were in excess of the number of C5 molecules, the number of lesions was determined by the number of C5 molecules. These data suggested, therefore, a spatial relationship of cell-bound C2, C4 and C5 molecules to the complement-dependent ultrastructural lesions. Further, in preliminary studies utilizing ferritin-conjugated antibody to C5, the ferritin marker was found to be primarily localized in the center of or adjacent to the ultrastructural lesions.

It has previously been demonstrated that only 1 of 20 cell-bound C4 molecules registers as an "effective molecule" (5) in the sense of the one-hit theory of immune hemolysis (4). In contrast, the present data demonstrate that each bound C4 molecule is capable of initiating the production of an ultrastructural membrane lesion. Similarly, although 3–12 cell-bound C5 molecules are required to produce one effective molecule (28), 1 in 2 of these molecules is capable of evoking the characteristic lesion. Further, the complexes EAC1-5, EAC1-6, and EAC1-7 (but not EAC1-3) possessed approximately the same number of ultrastructural lesions. However, the rates of lysis induced by the terminal complement components differed markedly between these complexes. With EAC1-7, the rate of lysis was rapid and complete; with EAC1-5, lysis was slow and incomplete. The difference in lysis of such complexes is known to be due to the decay of C5 sites on EAC1-5 prior to the addition of C6 and C7 (28). It may be concluded, therefore, that the ultrastructural lesions are distinct from the functional lesions which are responsible for cell lysis. Whereas the functional lesions are caused by C8 (29, 12), the ultrastructural lesions appear to be produced by

C5. The ultrastructural lesions are caused by C5 irrespective of the mechanism of its activation. In classical immune hemolysis C5 is activated by cell-bound $\overline{C4,2,3}$. By the recently described nonimmune mechanism, it is activated by fluid-phase $\overline{C4,2,3}$ and bound to cells lacking antibody, C1, C2, C4, and C3. The data further indicate that the ultrastructural lesions are unaffected by the decay of the hemolytic activity of bound C5, a process which, nevertheless, markedly reduces the number of functional lesions.

The question arises, is the ultrastructural lesion a membrane defect, or is it a manifestation of a characteristic assembly of complement molecules? Humphrey, Dourmashkin, and Payne (1) have shown that treatment with trypsin or acid pH in order "to remove antibody and/or C′ components" did not remove the ultrastructural lesions. However, conditions of treatment were not given nor was evidence supplied for removal of any complement proteins. It has been shown previously in this laboratory (30) that up to 95% of the cell-bound C3 can be removed by trypsin treatment of the cell complex. In the present study, when 95% of C3 and 100% of C5 were removed from the corresponding red cell intermediates by trypsin treatment, it was found that, as in the experiments of Humphrey et al. (1), the ultrastructural lesions were not removed; in fact, their appearance was clarified due to the removal of background negative-staining material. Thus it may be concluded that the ultrastructural manifestation of the complement reaction is truly a reflection of a membrane alteration and not due to an accumulation of exogenous protein on the surface of the red cell.

The relevance of the ultrastructural lesion to the functional lesion in the red cell which results in cell lysis still remains to be explained. It is apparent that the ultrastructural lesion itself is inert in producing cell damage. However, the possibility exists that it could be a preselected site for the action of C8. Whereas C8 may have the potential to interact at the site of each ultrastructural lesion, its interaction at only one site may be sufficient to damage the cell with the resultant defect in osmotic regulation leading to death of the cell and liberation of its macromolecules.

SUMMARY

A direct quantitative relationship has been demonstrated between the number of cell bound $\overline{C4,2}$ complexes or C5 molecules and the number of ultrastructural lesions visualized on the cell membrane subsequent to immune hemolysis. When bound $\overline{C4,2}$ complexes exceeded bound C5 molecules, the number of ultrastructural lesions seen corresponded to the number of C5 molecules. However, in the reverse situation, with bound C5 molecules in excess of bound $\overline{C4,2}$ complexes, the latter determined the number of lesions. During the complement-reaction sequence, the lesions first became visible in the nonlytic intermediate complex EAC1,4,2,3,5 and their number was unaffected when lysis was induced by C6–C9. Since the lesions were also demonstrable on the intermediate complex EC5,6,7, it is concluded that the protein C5 is responsible for their production. Once formed, the physical presence of the C5 molecule is no

longer required for the manifestation of the lesions as indicated by persistence of lesions after removal of C5 protein by trypsin. The C5-dependent ultrastructural phenomenon has therefore been interpreted to represent a true structural change of the membrane which, however, is not accompanied by a permeability defect.

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