MOLECULAR ANALYSIS OF THE MEMBRANE ATTACK MECHANISM OF COMPLEMENT*‡

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The attack mechanism of complement (1) has been defined as those complement proteins the binding of which to the target membrane is necessary for the production of membrane damage. It is distinguished from the recognition and activation mechanisms of complement which, although capable of attaching to the target cell, may act from the fluid phase or from the surface of another cell. Earlier work (2) demonstrated that the attack mechanism is comprised of five different components, namely C5,[1] C6, C7, C8, and C9. The present study was designed to obtain information which pertains to the molecular arrangement of these five proteins on a membrane under attack by complement. The accumulated evidence strongly suggests that the membrane-bound attack mechanism consists of a compact decamolecular assemblage with a total molecular weight of approximately 1 million.

Previous investigations have shown a requirement of protein binding to the target cell for expression of hemolytic activity for C5 (3–5), C6 (6, 7),[2] C8(8), and C9 (9). They have also pointed out a functional interdependence between C5, C6, and C7 (3), particularly in the formation of EC5, 6, 7[3] (2, 6). In addition, these three proteins were found to have an affinity for each other in free solution, enabling them to enter into reversible protein-protein interactions (3).[2] Such interaction has recently been demonstrated to occur also between C8 and C9.[4] Thus information published to date raised

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[1] Terminology employed conforms with the recommendations of the World Health Organization Committee on Complement Nomenclature (1968. *Bull. World Health Organ.* **39**:939).

[2] Arroyave, C. M., and H. J. Müller-Eberhard. Interaction of human C5 and C6 and their functional significance. Submitted to *Immunochemistry*.

[3] *Abbreviations used in this paper:* BSA, bovine serum albumin; CH_{50}, serum dilution at which 50% of the indicator red blood cells are hemolyzed; CM, carboxymethyl; E, sheep erythrocytes; EA, antibody-sensitized sheep erythrocytes; EAC, erythrocyte antibody complement; GVB, isotonic Veronal-buffered saline containing 1.5×10^{-4} M $CaCl_2$, 5×10^{-4} M $MgCl_2$, and 0.1% gelatin; TEAE, triethylaminoethyl.

[4] Haxby, J. A., and H. J. Müller-Eberhard. Manuscript in preparation.

the possibility of the formation of a macromolecular assembly of C5-9 on the surface of a target cell. Simultaneous experimentation with all five proteins was necessary, however, to deduce the molecular concept advanced below.

Materials and Methods

Human Complement Components and Complement Reagents.—Partially purified macromolecular C1 was prepared from human serum according to Nelson et al. (10). C2 was purified as described previously (11) and was used exclusively in its oxidized form (12). C3 (13), C4 (14), C5 (13), and C6 (15) were obtained according to published methods. C7 was isolated in highly purified form according to a method which will be described in detail elsewhere.[5] Highly purified C8 and C9 were prepared from the same pool of human serum by modifications of the previously published methods (8, 9). These modifications will be presented in detail elsewhere.[6] The C8 and C9 thus prepared were judged free of demonstrable contamination by chromatographic, electrophoretic, and immunochemical criteria. Protein concentrations of the purified components were determined by the Lowry method (16) using the following standards: C3 for C3, C6, and C7; C4 for C4; C5 for C5; and human Cohn fraction II for C8 and C9.

Complement components used for quantitative uptake experiments were trace labeled with either ^{125}I or ^{131}I by the chloramine-T method (17). After labeling, each component was dialyzed for 48 hr against 4 × 10 liters of 0.15 M NaCl containing 50 mM chloramphenicol (donated by Parke, Davis & Company, Detroit, Mich.) and 25 μM kanamycin sulfate (Bristol Laboratories, Syracuse, N.Y.). The dialyzed preparations were centrifuged for 40 min at 45,000 *g* to remove aggregates. The hemolytic activities of C5 and C9 were essentially unchanged by labeling, while C6 and C8 lost maximally 30–40%. The specific radioactivities of the preparations used for experiments reported in this communication were: C5, 4.5–22 × 10^5 cpm/μg; C6, 6.5–8 × 10^4 cpm/μg; C8, 1.44–3.94 × 10^6 cpm/μg; C9, 1.65–4 × 10^6 cpm/μg. Bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) was added as carrier to a final concentration of 1 mg/ml and the preparations were stored at 0°C.

A reagent containing C3, C6, and C7 was prepared by carboxymethyl (CM)-cellulose column chromatography by a minor modification of the published method (8): only the fractions corresponding to the peak of the breakthrough protein were pooled and the pH was adjusted to 7.0 with 1 M NaOH before freezing.

Preparation of Sheep Erythrocyte-Antibody-Complement Complexes.—EAC1,4,^{oxy}2 was usually prepared from iodinated human serum (18) and phloridzin: 175 mg of phloridzin (K & K Laboratories Inc., Plainview, N.Y.) was dissolved in 15 ml of isotonic Veronal-buffered saline (GVB) by briefly heating to 70°C. After rapid cooling to approximately 40°C, the phloridzin solution was mixed with an equal volume of sensitized sheep erythrocytes (5 × 10^8 cells/ml) prewarmed to 37°C. After 1 min, 2 ml of the iodinated serum was added and the mixture was incubated at 37°C for 14 min with periodic agitation. The reaction mixture was then added to 90 ml of ice-cold GVB, centrifuged, washed twice in 50-ml portions, and resuspended in GVB to a final cell concentration of 5 × 10^8 cells/ml. These cells were usually employed for the preparation of EAC1-7 as follows. To 15 ml of EAC1,4,^{oxy}2 (5 × 10^8 cells/ml) was added a mixture of 1 ml of the C3, C6, C7 reagent, 200 μl of 40% glucose, and 200 μg of C5. This mixture was incubated 45 min at 37°C, centrifuged, washed three times with 50 ml of cold GVB, and resuspended in GVB to the appropriate cell concentration. For the experiments involving determination of the molar C5/C6 ratio on EAC1-7, the EAC1,4,^{oxy}2,3 cells were prepared from EA and purified components (22).

EAC1-8 was prepared by incubation of EAC1-7 (6 × 10^7 cells/ml) with an appropriate

[5] Arroyave, C. M., and H. J. Müller-Eberhard. Manuscript in preparation.

[6] Haxby, J. A., W. P. Kolb, and H. J. Müller-Eberhard. Manuscript in preparation.

amount of C8 for 30 min at 37°C. The cells were then centrifuged and washed three times with portions of GVB equal to one-half the original volume.

Antisera.—Monospecific antisera to C3, C5, C6, C7, and C8 were produced in rabbits by injection into the popliteal lymph nodes according to Goudie et al. (19). For C3, C6, C7, and C8, the protein band was sliced from polyacrylamide disc electrophoresis gels. The gel slices were crushed and mixed with an equal volume of Freund's complete adjuvant and 100 μl of the mixture containing 20 μg protein was injected into each lymph node. 1 month later 50 μg of protein in 200 μl of a similar mixture was injected intramuscularly into each of two sites in the hindquarters. The rabbits were bled 6–8 days later. The same procedure was followed for C5, except the protein was not sliced from polyacrylamide gels but injected with an equal volume of Freund's complete adjuvant only.

Purified γ-globulin fractions of each antiserum and normal rabbit serum, free of rabbit C8 and C9, were prepared as follows. 20 ml of each serum was dialyzed 24 hr against 10 liters of cold sodium-phosphate buffer, pH 7.0, conductivity 1.5 mmho/cm. After removal of insoluble protein by centrifugation at 3000 *g* for 30 min, the dialyzed sample was applied to a 3 × 60 cm triethylaminoethyl (TEAE) column equilibrated with the dialysis buffer and the breakthrough protein was collected and concentrated by precipitation at 0°C with ammonium sulfate at 50% saturation. The precipitate was washed three times with 50 ml of 50% saturated ammonium sulfate, dissolved in and dialyzed against 0.15 M NaCl (2 × 10 liters for 2 days at 4°C), heated at 56°C for 2 hr, cleared of aggregates by centrifugation (as above), and stored at −70°C.

C8 and C9 Hemolytic Assays. The samples to be assayed for C8 activity were incubated with 3×10^7 EAC1-7 and an excess (100 CH_{50}) of C9 at 37°C in a final volume of 500 μl of GVB. The reaction was terminated (usually after 30 min) by transferring the tubes to an ice bath, addition of 1 ml of ice-cold 0.15 M NaCl, and immediate centrifugation. Oxyhemoglobin in the supernatants was quantitated by reading the absorbance at 412 nm. The results were corrected for controls in which GVB was substituted for the sample. C9 hemolytic activity was quantitated by the same method, except 100 CH_{50} of C8 replaced the C9.

Determination of the Molar Ratio of Cell-Bound C5 and C6 on EAC1-7.—Various amounts of C6-^{131}I (200 ng–10 μg) and a constant amount of C5-^{125}I (10 μg) were incubated for 20 min at 37°C with 2.5×10^7 EAC1-3 cells in the presence of C7 (10 μg). The total reaction volume was 300 μl. After incubation the tubes were centrifuged and the cells washed four times with 5-ml portions of GVB at room temperature. The final cell buttons were resuspended in 500 μl of GVB, transferred to another set of tubes, and analyzed for radioactivity in a well-type scintillation counter. The cells were then hemolyzed by the addition of 2.5 ml of water, the absorbance of the lysate was read at 541 nm, and the data were normalized to counts per minute per 2.5×10^7 cells. Specific C6-^{131}I and C5-^{125}I uptakes were calculated by subtracting the nonspecific uptake of these components by EAC1,4. The molar ratios of the specifically bound C5-^{125}I to C6-^{131}I were calculated by assuming a molecular weight of 180,000 for C5 and 125,000 for C6.

Determination of Molar Ratio of Bound C8 and Bound C5 on EAC1-8.—Three populations of radiolabeled EAC1-7 cells were made by reacting 1.75×10^9 EAC1,4, ^{oxy}2 cells with 2.5 μg of C5-^{125}I and respectively with 250, 500, and 750 μl of the C3, C6, C7 reagent in a final volume of 5.0 ml. The amount of C5-^{125}I uptake was observed to remain constant with amounts of C3, C6, C7 reagent greater than 400 μl, thus indicating an excess of C6 and C7. The radiolabeled EAC1-7 cells were washed four times in 10 ml of 4°C GVB and 1.5×10^8 cells were placed in each of nine tubes. One tube served as a control from which the number of C5-^{125}I molecules bound per cell was calculated. The other tubes in each series received C8-^{125}I ranging from 150 to 3500 C8 molecules offered/cell. The reaction volume was 900 μl and the incubation was for 45 min at 37°C with continuous shaking. The cells were diluted and washed four times in 10 ml of GVB at room temperature and the tubes were briefly inverted on absorbent tissue to blot to dryness each time. The final cell pellet was resuspended

in 500 μl of GVB, counted as described above, and the counts were normalized to 1.5×10^8 cells. Nonspecific uptakes of C5-^{125}I and C8-^{125}I were obtained in all experiments by treating a similar number of EAC1,4,^{oxy}2 with the appropriate amounts of the labeled component in parallel with the experimental incubations. The specific C8-^{125}I uptake was calculated by subtracting the total C5-^{125}I uptake and the nonspecific C8-^{125}I uptake from the total counts. For calculation of the molar ratio of the specifically bound C5-^{125}I to C8-^{125}I, the molecular weight of C8 was assumed to be 153,000 and that of C5, 180,000. Alternatively, differential labeling was used, i.e., C5-^{125}I and C8-^{131}I.

Determination of Molar Ratio of Bound C9 to Bound C8 on EAC1-9.—6 ml of EAC1-7 (5×10^8 cells/ml) was incubated with 1 μg of C8-^{125}I for 60 min at 37°C. The cells were washed four times with 10 ml of GVB at room temperature and 1×10^8 cells were placed in each of nine tubes. One tube served for determining the number of C8 molecules bound per cell and the others received C9-^{125}I ranging from 200 to 19,600 molecules offered/cell. Incubations were in a total volume of 900 μl for 45 min at 37°C with continuous shaking. The reaction mixture was diluted to 5 ml with GVB and the cells and stroma were collected on Millipore filters (Millipore Corp., Bedford, Mass.) as described below. Nonspecific uptake of C8-^{125}I was determined by using EAC1,4,^{oxy}2 and of C9-^{125}I by using EAC1-7 (always from the same stock cell suspension which had been used to prepare the radiolabeled EAC1-8).

The Millipore filters used in these experiments were 25 mm in diameter, pore size 0.45 μ (Millipore No. HAWP025), and were soaked for at least 12 hr in 1% bovine serum albumin (BSA) to minimize nonspecific uptake of ^{125}I-labeled proteins. To collect a sample a filter was placed in a collecting apparatus (similar to Millipore No. XX10 025 00) and the vacuum was applied. The vacuum employed was provided by a water aspirator and was manually regulated by use of a "bleeding" valve in the vacuum line, thereby insuring a smooth and gentle washing action. The cell or cell-stroma suspension to be collected was placed in the collecting apparatus and allowed to drain to a volume of about 0.5 ml, at which time 5 ml of distilled water were added. This converted any unlysed cells to stromata. After the water had drained to approximately 1 ml, 4 ml of 0.15 M NaCl was added and the apparatus was drained to dryness. Next the stroma were washed with 2–5 ml portions of 0.15 M NaCl and the filters were placed in 12 × 75 mm tubes and counted in a well-type scintillation counter.

This procedure was validated by preliminary experiments which indicated that the filtration and washing resulted in minimal loss of C8-^{125}I from EAC1-8 and EAC1-9 within a wide range of C8 and C9 multiplicities. Thus, the filters were able to quantitatively retain the fragments of complement-lysed cell membranes, even when 100% lysis of EAC1-9 had occurred, and, in addition, the bound C8 (and presumably the C9) was not washed from these fragments.

The specific C9-^{125}I uptake was calculated by subtracting the total C8-^{125}I uptake and the nonspecific C9-^{125}I uptake from the total counts. For calculations of the C9-^{125}I–to–C8-^{125}I molar ratio on the cell surface, the molecular weight of C9 was assumed to be 79,000. In some experiments a differential labeling technique was used: C8-^{125}I and C9-^{131}I.

Inhibition of C8 Uptake and Hemolysis of EAC1-7 and C9 Uptake and Hemolysis of EAC1-8 by Various Antisera.—5×10^8 EAC1-7 or EAC1-8 cells in 500 μl of GVB were added to 2.0 ml of GVB containing increasing amounts of γ-globulin fraction from either antiserum (anti-C3, -C5, -C6, -C7, -C8, or -Forssman) or normal rabbit serum. After incubation for 15 min at 37°C, 5 ml of 4°C GVB was added, the tubes were centrifuged, and the cells were washed three times with 5-ml portions of the same buffer. The washed cell pellets were resuspended to a concentration of 5×10^8 cells/ml. The residual hemolytic potential of the cell-bound complement components was found by incubation of the antibody-treated EAC1-7 with 2 CH_{50} units of C8 and excess C9 and the antibody-treated EAC1-8 with 2 CH_{50} units of C9. The hemolytic reactions were stopped at 40–50% hemolysis in the control incubations, a value well within the linear range of the reaction. To ascertain the effect of antibody treatment on C8 or C9 binding, 1.5×10^8 of the treated EAC1-7 or EAC1-8 cells were incubated

40 min at 37°C with C8-^{125}I or C9-^{125}I, respectively. The selected dose of radiolabeled C8 or C9 was determined from preliminary experiments to be favorably situated on the linear portion of the uptake curve for cells which had not been exposed to rabbit γ-globulin (GVB cells). The uptakes of the C8-^{125}I and C9-^{125}I were quantitated by the same methods described in the sections on molar ratios of cell-bound C5 to C8 and C8 to C9, respectively. The results are expressed as per cent of normal rabbit γ-globulin controls which showed essentially constant hemolysis and uptake of radiolabeled C8 and C9 over the protein range studied and differed by no more than a few per cent from GVB cells.

Effect of Various Antisera on Persistence of Cell-Bound C5 and C8.—EAC1-7 were made with C5-^{125}I as described in the section on the molar ratio determination of C5 to C8, and EAC1-8 were made with C8-^{125}I as described in the section on the molar ratio determination of C8 to C9. The cells were washed four times with 10 ml of GVB at room temperature and 1.5×10^8 of the EAC1-7 or EAC1-8 cells were placed in a series of tubes and incubated with GVB or 250 μg (50 μg/3 × 10^7 cells) of the γ-globulin fraction from normal rabbit serum, anti-C3, -C5, -C6, -C7, or -C8 in a total volume of 750 μl for 30 min at 37°C. The cells were washed three times with GVB at room temperature and the bound C5-^{125}I or C8-^{125}I was quantitated by the usual methods described in other sections.

Microtiter Procedures.—Microtiter plates and microtiter dilutors (Cooke Engineering Co., Alexandria, Va.) were used for the titration of the various antibodies employed in these studies. 25 μl of GVB were placed in each well and 25 μl of the γ-globulin fraction to be tested were delivered to the first well of the series After doubling dilutions were made by employing 25-μl microtiter dilutors, each well received 25 μl of the appropriate cell suspension which was at a concentration of 10^8 cells/ml. After the plates were agitated and stored at 37°C for 30 min they were agitated again and stored at 4°C overnight and the settling pattern was recorded.

Analysis of C9 Hemolytic Dose Response and Binding Reactions.—A tube containing 5 ml of EAC1-7 (5 × 10^8 cells/ml) was incubated with 0.1 μg of C8-^{125}I (4.5 × 10^5 cpm/μg) for 20 min at 37°C. The radiolabeled cells were washed four times in 10 ml of GVB at room temperature and 5 × 10^7 cells were placed in a series of eight tubes. The first tube in each series was used to determine the number of C8-^{125}I molecules bound per cell. The other tubes received amounts of C9-^{125}I (1.65 × 10^6 cpm/μg) ranging from 25 to 1680 molecules offered per cell. The cells were incubated in a total volume of 500 μl for 60 min at 37°C with continuous shaking. The reaction mixtures were then diluted to 2.5 ml with 0°C GVB, centrifuged, and the extent of lysis was quantitated by determination of the absorbance of the supernatants at 412 nm. The cell pellets and supernatants containing cell stroma were quantitatively collected on Millipore filters and counted as described above. The specific C9-^{125}I uptake was also calculated as described above.

RESULTS

Molecular Ratios of Attack System Components on the Target Cell Surface.—A prerequisite for the formulation of a molecular model of the attack system was the determination of the stoichiometry of these cell-bound complement molecules. Measurement of the C5/C6 ratio was approached in three different ways. One series of experiments was conducted by offering EAC1,4,^{oxy}2,3, a constant amount of C5-^{125}I, various amounts of C6-^{131}I, and an excess of unlabeled C7. The cells were washed and the quantities of bound C5 and C6 were measured (Fig. 1). The supernatants from these reaction mixtures, upon incubation with E, yielded EC5,6,7[2] and the molar ratio of bound C5 and C6 was determined. In another series of experiments,[2] a hemolytically active radio-

labeled $\overline{C5,6}$ complex was generated in solution by reacting C5-^{125}I and C6-^{131}I in the presence of trypsin.[2] After addition of soybean trypsin inhibitor, E were treated with the reaction mixtures plus C7. The cells were washed and bound C5 and C6 were determined. The data obtained in all three types of experiments led to the conclusion that the molar ratio of cell-bound C5 and C6 was approximately 1.0 and that it was independent of the absolute and relative amounts of C5 and C6 input and uptake.

In order to determine the molar ratio between cell-bound C5 and C8, EAC1-7 was prepared which had known numbers of C5-^{125}I molecules per cell. In a typical experiment EAC1-7 with 157, 185, and 191 C5 molecules (and presumably

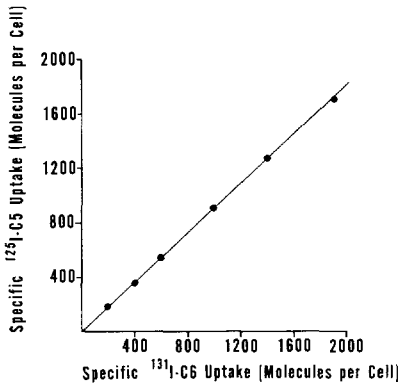

FIG. 1. Demonstration of proportionality between C5 and C6 uptake by EAC1,4,^{oxy}2,3 and of an equimolar ratio of cell-bound C5 and C6. 10 μg of C5-^{125}I and 200 ng–10 μg of C6-^{131}I were incubated with 10 μg of C7 and 2.5 $\times$ 10^7 EAC1,4,^{oxy}2,3 cells for 20 min at 37°C in a total volume of 0.3 ml. The cells were washed and the number of specifically bound C5 and C6 molecules per cell was determined. The C5/C6 ratio on the cell surface was constant over a wide range of C6 input.

C5,6,7 sites) per cell were incubated with increasing amounts of C8-^{125}I and the specific C8 uptake was determined. The results (Fig. 2) indicate that the C8/C5 ratio on the target cell surface increased with C8 input and approached 1.0 asymptotically.

The molar ratio of cell-bound C8 to C9 was obtained by using EAC1-8, made with C8-^{125}I, and radiolabeled C9. Fig. 3 depicts the results of a typical experiment in which EAC1-8 possessing 109 C8 molecules per cell was reacted with increasing amounts of C9-^{125}I. The molar C9/C8 ratio increased with C9 input and at saturation of C9 binding sites approached 6.

Studies of the Spatial Arrangement of C5-C9 on the Target Cell Surface Using Specific Antibodies to Complement Proteins.—The availability of specific antisera to various human complement proteins provided a means for the exploration of the spatial arrangement of cell-bound C5–9. In one series of experiments

EAC1-7 cells were treated with the γ-globulin fraction of a monospecific rabbit antiserum to either C3, C5, C6, C7, or Forssman antigen. These cells were then tested for their ability to bind radiolabeled C8 and to undergo lysis in the presence of limited amounts of C8 and an excess of C9. As depicted in the top half of

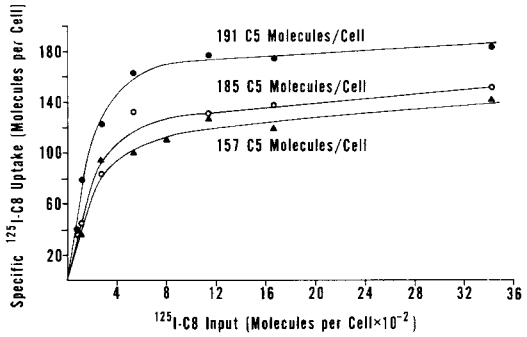

FIG. 2. Demonstration of equimolarity between bound C8 and bound C5 at saturation of C8 binding sites on EAC1-7. Relationship between specific uptake of C8 and C8 input for three cell preparations which differed in the number of specifically bound C5 molecules per cell. The reaction mixtures contained 1.5 × 10^8 EAC1-7 cells and the indicated amount of C8-^{125}I in a volume of 0.9 ml and were incubated for 45 min at 37°C.

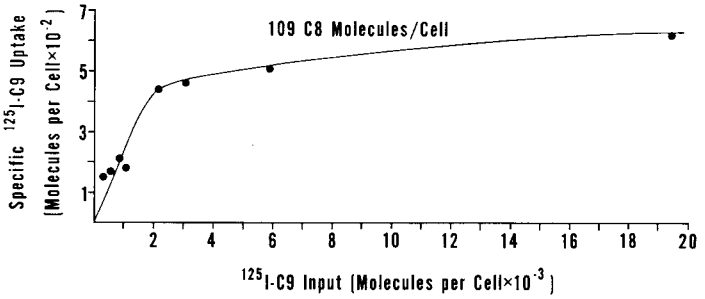

FIG. 3. Binding of multiple C9 molecules per C8 molecule on EAC1-8. Relationship between specific uptake of C9-^{125}I and C9 input using EAC1-$\bar{6}$ with 109 C8 molecules per cell. The reaction mixtures contained 1.0 × 10^8 cells and the indicated amount of C9-^{125}I in a volume of 0.9 ml and they were incubated for 45 min at 37°C before the lysed and unlysed cells were collected and washed on Millipore filters.

Fig. 4, antibody to C5, C6, or C7 inhibited C8 uptake, whereas anti-Forssman and anti-C3 had no significant effect. As shown in the bottom half of Fig. 4, antibody to C5 or C6 also inhibited lysis of EAC1-7 while anti-Forssman was ineffective. Although higher levels of anti-C3 were apparently slightly inhibitory, this cannot be assigned significance because of intense agglutination of the cells (see below). In contrast to the above antisera, anti-C7 gave somewhat incon-

sistent results; while it inhibited C8 uptake in every experiment, inhibition of hemolysis was not always demonstrable and was at best marginal.

In a second series of experiments EAC1-8 cells were treated with the γ-globulin fraction from the above antisera or from anti-C8. The antibody-treated cells

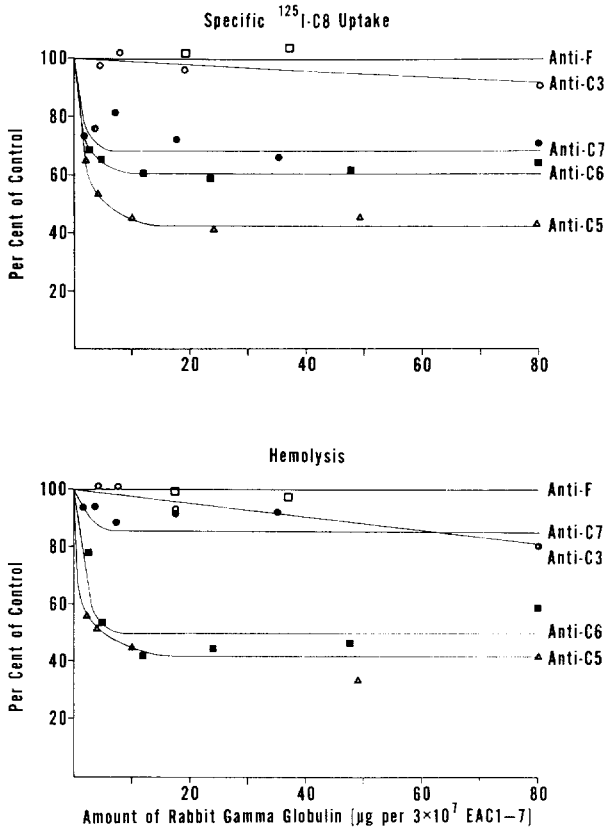

FIG. 4. Inhibition by various complement antisera of the interaction between EAC1-7 and C8. EAC1-7 were pretreated with increasing amounts of the γ-globulin fraction from various antisera, or normal rabbit serum, and assayed for their ability to bind radiolabeled C8 and for their susceptibility to hemolysis by limited amounts of C8 and excess C9. The results are expressed as the per cent of the average hemolysis or C8 binding obtained with normal rabbit γ-globulin treated cells, which were assigned a value of 100%. The control cells themselves gave 65% hemolysis and C8-^{125}I binding of 3480 cpm/1.5 × 10^8 cells.

were assessed for their ability to bind C9 and to undergo hemolysis by C9. The results of a representative binding experiment are illustrated in the upper half of Fig. 5. They indicate that antibody to C5, C6, C7, or C8 can inhibit the binding of C9 to EAC1-8, anti-C8 always producing the greatest degree of inhibition of the antisera tested. As shown in the lower half of Fig. 5, hemolysis by

C9 was strongly inhibited by anti-C8 and anti-C5. However, it was unaffected by anti-C6 or anti-C7, although both antisera reduced C9 binding. This apparent discrepancy will be explained in the Discussion.

Inhibition of C8 and C9 binding by the various antibodies was most probably

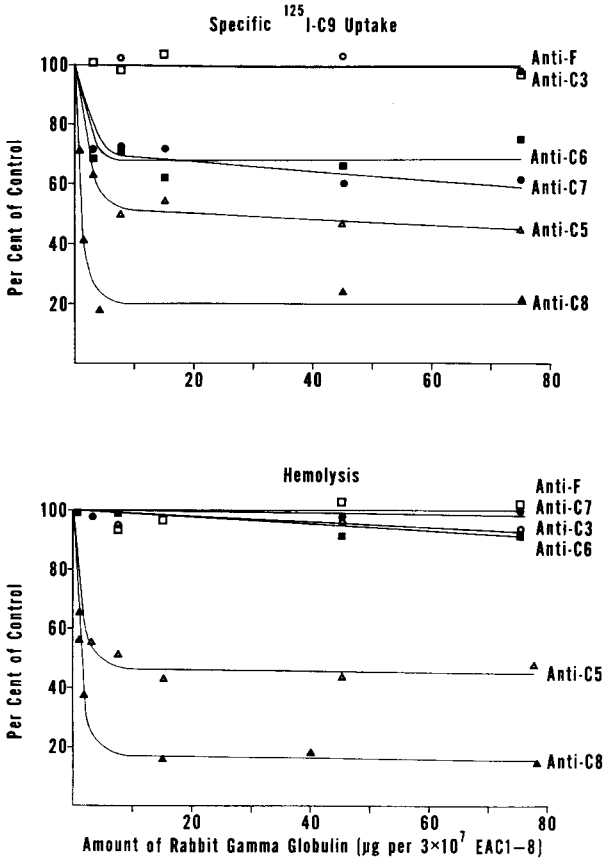

FIG. 5. Inhibition by various antisera of the interaction of EAC1-8 with C9. EAC1-8 were pretreated with the various rabbit γ-globulin fractions as described in the legend of Fig. 4, with the addition of anti-C8, and assayed for their ability to bind radiolabeled C9 and for their ability to undergo hemolysis by limiting C9. The results are expressed as in Fig. 4. The control cells gave 44% hemolysis and C9-^{125}I binding of 6044 cpm/1.5 × 10^8 cells.

due to sterical hindrance imposed by the antibodies. Before this conclusion could be drawn it was necessary to show that treatment with antibody caused no dissociation of complement molecules from the target cell surface. The data in Table I indicate that both C5-^{125}I on EAC1-7 and C8-^{125}I on EAC1-8 remained associated with the cells upon treatment with the antibodies. It was also necessary to test whether the antibody-dependent inhibition was a function of the

TABLE I

Persistence of Cell-Bound C5 and C8 on Cells Treated with Various Complement Antisera

Cell type	Residual radioactivity after treatment with:						
	GVB	Normal rabbit γ-globulin	Anti-C3	Anti-C5	Anti-C6	Anti-C7	Anti-C8
EAC1-7 (C5-^{125}I)	20,400	20,200	21,500	19,700	21,200	19,270	N.D.
EAC1-8 (C8-^{125}I)	8930	8500	9000	9100	9280	8840	8900

Treatment: 50 μg γ-globulin fraction per 3 × 10^7 cells, 15 min, 37°C. Radioactivity is expressed as counts per minute per 1.5 × 10^8 cells.

TABLE II

Agglutination of Complement-Erythrocyte Intermediates by Complement Antisera

Cell type	Minimum agglutinating dose (micrograms per 3 × 10^7 cells) of γ-globulin fraction of anti:					
	Forssman	C3	C5	C6	C7	C8
EAC1-3	7.1	3.2	–	–	–	–
EAC1-7	7.1	3.2	1.5	0.7	0.2	–
EAC1-8	7.1	3.2	1.5	0.7	0.1	1.4

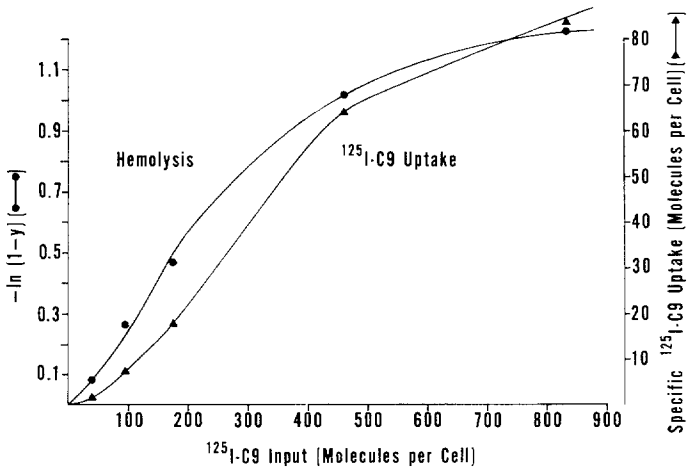

FIG. 6. Demonstration of sigmoidal binding and hemolytic dose response curves for C9. EAC1-8 possessing 59 C8 molecules per cell were offered increasing amounts of C9-^{125}I and the extent of hemolysis recorded. The stroma and remaining cells were collected on Millipore filters and the C9-^{125}I specifically bound per cell was determined.

degree of cellular agglutination. From Table II and Figs. 4 and 5 it is apparent that there was no correlation between the minimum agglutination dose and the degree of inhibition. Moreover, anti-C3 which produced strong agglutination

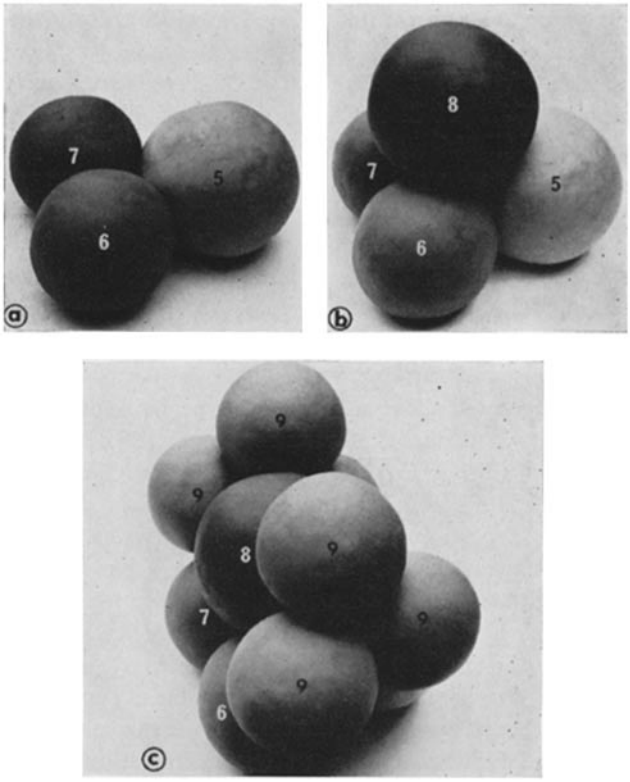

FIG. 7. Photograph of a model of the decamolecular membrane attack mechanism of complement showing three stages of its assembly. The spheres were made of modeling clay, the relative weights being proportional to the molecular weights of the complement proteins C5–9. The numerals refer to the corresponding complement components. (*a*) Model of the membrane-bound C5, 6, 7 trimolecular complex displaying triangular geometry, and constituting the proposed binding site for C8. (*b*) Model of the tetramolecular complex C5, 6, 7, 8 having the geometry of a tetrahedron. (*c*) Model of the fully assembled decamolecular C5–9 complex, exhibiting two C9 trimers bound symmetrically in triangular arrangement to the C8 portion of the tetrahedron (the C9 trimer on the backside of the complex is only partially visible).

(complete settling of the cells within 5 min) had little effect upon the hemolytic and binding reaction of C8 and C9. Anti-C8, on the other hand, was a weak agglutinator of EACl-8 but a potent inhibitor of C9 uptake and hemolysis.

Since the above data suggested that the C8 binding site was on or near C5, C6, and C7, the question arose as to whether binding of C8 to EAC1-7 influenced

their agglutination by anti-C5, anti-C6, or anti-C7. The data (Table II) indicate that these antibodies agglutinated both EAC1-7 and EAC1-8 to about the same degree. Therefore, the C8 binding apparently does not completely obscure C5, C6, or C7 from availability to antibody. This is in agreement with the hemolytic and binding experiments presented in Fig. 4, in which these antisera showed maximal inhibition of about 50–60%.

Comparative Analysis of C9 Binding and of C9-Dependent Hemolysis.—The multimolar ratio of cell-bound C9 to C8 raised the possibility that C9 uptake is a cooperative process. Therefore, experiments were initiated to establish a C9 binding curve and to compare it with a C9 hemolytic dose response curve. EAC1-8 cells possessing 59 C8 molecules per cell were reacted with increasing amounts of C9-^{125}I. As seen in Fig. 6, both curves showed sigmoidal behavior, indicating cooperativity in C9 binding and the requirement of more than one molecule of C9 for achievement of a lytic effect.

DISCUSSION

From the results presented above and from other available data, the following tentative concept may be deduced. After activation of C5, i.e., cleavage of the molecule into C5a and C5b by $\overline{C4,2,3}$ (1), C5b, C6, and C7 are bound to the surface of a membrane in the form of a complex. The simplest and most probable geometry of this trimolecular complex is a triangular arrangement, each component contributing one molecule to the triangle. Since the molecular weight of C5b is 165,000 and that of C6 and C7 each approximately 100,000, the particle weight of the complex is about 365,000. The central region of the triangle is thought to constitute the binding site for C8. It is formed by limited portions of the surface of each of the three molecules and is capable of accommodating one C8 molecule, which is bound by adsorption without prior enzymatic modification. The proposed tetramolecular complex has the geometry of a tetrahedron. The bound C8 molecule is endowed with multiple binding sites for C9 and can maximally accommodate six molecules which are attached cooperatively by an adsorption process. Since the molecular weight of C8 is 150,000 and that of C9 is 80,000, the molecular weight of the fully assembled complex, $C5b_1C6_1C7_1C8_1C9_6$, equals 995,000. Fig. 7 is a photograph of a simple three dimensional model of the three assembly stages of the C5–9 complex.

The proposed molecular concept is predicated essentially on three hypotheses: (*a*) that at the target membrane surface C5, C6, and C7 are bound in close spatial proximity to each other, allowing mutual physical contact; (*b*) that the C5,6,7 complex constitutes the binding site for C8; and (*c*) that C8 bears multiple binding sites for C9.

The hypothesis that C5, C6, and C7 are bound to the target cell surface as a complex is supported by the following observations. Both C6 and C7 exhibit stereochemical affinity for C5 as was demonstrated by sucrose density gradient ultracentrifugation of mixtures of the three proteins in their native form (3).[2]

Whereas the complex of the native proteins is readily reversible, C5 and C6 have been shown to form a stable complex, $\overline{C5,6}$ (6),[2] when acted upon by $\overline{C4,2,3}$ or trypsin.[2] And whereas $\overline{C5,6}$ alone is unable to attach itself to a cell (E), binding to E occurs in the presence of and together with C7 (6).[2] Further, formation of EC5,6,7 by fluid phase $\overline{C4,2,3}$ requires the simultaneous presence of C5, C6, and C7; an EC5 or EC5,6 intermediate complex was not observed (2). In contradistinction, C5 may be bound to EA$\overline{C4,2,3}$ (4, 5); however, the cytolytic activity of the bound molecule decays with a half-life of 2 min at 37°C unless C6 and C7 are offered (4).

The hypothesis that C5, C6, and C7 contribute jointly to the formation of the C8 binding site is supported by these observations. The hemolytically effective binding of C8 to a membrane requires prior attachment of C5, C6, and C7. Uptake of C8 by EAC4-7 is inhibited by pretreatment of the cells with anti-C5, anti-C6, or anti-C7, indicating that C5, C6, and C7 are so close to the C8 binding site that antibody molecules directed to any one of the three molecules impose a steric restriction on C8 uptake. In view of the ability of C8 to initiate a limited degree of membrane damage in the absence of C9 (20), the possibility was considered that C8 binds directly to the membrane, perhaps after enzymatic activation by $\overline{C5,6,7}$ and subsequent transfer to a membrane receptor. However, if such a mechanism were operative, enzymatic turnover of substrate (C8) should produce an amplification of uptake and inactivation of unbound molecules as was described for C2 (21), C3 (22), C4 (23), and C5 (4). These latter complement proteins are, in fact, bound only after enzymatic activation of their binding sites and transfer to the site of binding, and consequently their efficiency of binding is low (5–15%). In contrast, the efficiency of binding of C8 may be unusually high, under optimal conditions approaching 70% of input.[7] There is no amplification of uptake, since the maximal number of C8 molecules bound per cell approached but never exceeded the number of bound C5 molecules (and, by implication, that of bound C6 and C7 molecules). Nor is there detectable inactivation of unbound C8 before or after saturation of C8 binding sites. Also, uptake of C8 is largely independent of changes in pH, ionic strength, and temperature.[7] Together these observations are strongly indicative of uptake of C8 by adsorption to $\overline{C5,6,7}$ rather than enzymatically catalyzed binding to membrane constituents.

The hypothesis that C9 combines with the C8 portion of the tetramolecular complex, $\overline{C5,6,7,8}$, is supported by the fact that C9 does not attach to $\overline{C5,6,7}$ sites and that the number of C9 molecules bound per cell is governed solely by the number of C8 molecules. In accordance with this view is the observed inhibition of C9 uptake by pretreatment of EAC4-8 with antibody to C8. Also, in the case of C9, an enzymatic uptake mechanism may be envisaged (9). That 1 mole of bound C8 can effect uptake of 6 moles of C9 could be indicative of an

[7] Haxby, J. A., J. A. Manni, W. P. Kolb, and H. J. Müller-Eberhard. Manuscript in preparation.

enzymatic reaction. However, at 6 moles of C9/mole of C8, C9 uptake becomes completely independent of the C9 concentration in the reaction mixture. Loss of fluid phase C9 activity is satisfactorily accounted for by cellular C9 uptake, and no inactivation of unbound C9 is encountered after saturtation of all cellular binding sites.[8] These observations virtually preclude an enzymatically catalyzed binding reaction. Direct evidence for stereochemical affinity of native C9 for C8 comes from transport experiments in free solution utilizing density gradient ultracentrifugation.[4] From the model depicted in Fig. 7 it would appear that spatial considerations impose no restrictions on the association of six C9 molecules with one C8 molecule, even if the latter constitutes a subunit of a tetrahedron. This applies to two separate triangular arrangements, as depicted, as to the hexameric arrangements, namely the hexagon, trigonal prism, or octahedron (24), which are not shown.

The functional significance of multiplicity for expression of C9 hemolytic activity is manifested by the observed sigmoidal dose-response curve (lysis of EAC4-8 *versus* C9 input) (Fig. 6). According to this curve, more than one C9 molecule per C8 site is required for production of a lytic effect. In addition, the also sigmoidal binding curve (C9 uptake *versus* C9 input) (Fig. 6) indicates cooperative interaction of C9 molecules during the binding process. This means that binding of one C9 molecule facilitates uptake of other C9 molecules to the same C8 site. Or the affinity of C9 for C8 is low at low C9 concentrations and increases with partial saturation of C8. Thus, at partial saturation a significant number of C8 molecules may bear up to six C9 molecules and others none. It is not surprising therefore when in a given experiment 63% lysis [or $-\ln(1-y) = 1$] is achieved with an average molar ratio of bound C9 to bound C8 of one (Fig. 6). The number of bound C9 molecules required for production of a given degree of lysis was found to be inversely related to the number of C8 molecules on the cells. When a respective graphical plot (not shown) was extrapolated to very small numbers of C8 molecules per cell, the number of C9 molecules per C8 site, required to cause 63% lysis, approached three.[8] Thus, although one C8 molecule can bind maximally six C9 molecules, the binding of three C9 molecules per C8 molecule appears to be sufficient for the production of a full hemolytic effect. In the model shown in Fig. 7, the six C9 molecules are therefore represented in the form of two triangular arrangements bound symmetrically to C8. The proposed model may explain the differential effect of anti-C6 or anti-C7 on C9 binding and hemolysis: whereas either antibody should sterically interfere with the binding of one C9 trimer, it should not affect attachment of the other.

The data are in accord with a possible allosteric effector function of C9. The demonstrated ability of a small molecule such as 2,2′-bipyridine to substitute functionally for C9 further emphasizes the probability of an allosteric mecha-

[8] Kolb, W. P., J. A. Haxby, and H. J. Müller-Eberhard. Manuscript in preparation.

nism. The dose-response curve obtained with bipyridine was also sigmoidal (9). The chelating capacity of this compound for bivalent metal ions and the previously described inhibition of C9 by ferrous ions (25) strongly implicate a metal in the interaction of C8 and C9. Work is underway to examine this hypothesis.

One of the most intriguing questions, which presently remains unanswered, is how the decamolecular attack mechanism affects biological membranes. Several years ago, Fischer (26) speculated that the terminal complement component represents a phospholipase which by liberating lysolecithin initiates a nonenzymatic attack on membrane lipids. Inoue and Kinsky (27), working with artificial membrane systems containing radiolabeled phospholipids, found no evidence for enzymatic degradation of the latter by complement. They suggested that in analogy to the mode of action of the polyene antibiotics (28), complement causes membrane lesions by hydrophobic interaction with membrane lipids.

The characteristic morphological changes of membranes caused by complement (29) may well be due to a nonenzymatic, physicochemical effect of one of the complement proteins. Recently, these ultrastructural lesions were shown to be caused by bound C5 (30), and thus to be distinct from functional membrane lesions which permit uncontrolled flux of ions. It appears likely that, upon removal of C5a, C5b assumes transiently the characteristics of a detergent protein, displaying a strongly hydrophobic region with affinity for membrane phospholipids. Whereas the resulting rearrangement of membrane constituents affects only the outer layer of the membrane,[9] it is conceivable to us that complete assembly of the C5–9 complex leads to an augmentation of the detergent effect of C5 with conversion of an ultrastructural to a functional lesion. The manner in which C8, 9 produces a functional lesion, particularly when, as proposed in this paper, these proteins do not bind directly to the target membrane, remains to be explained.

SUMMARY

The molecular arrangement of the membrane attack mechanism of complement was explored. The molar ratios of the components within the C5-9 assembly on the target cell surface were determined using human complement proteins in highly purified and radiolabeled form. With the aid of monospecific complement antisera it was possible to probe the spatial relationships between the components of the assembly.

C5 and C6, in the presence of C7, were bound to EAC1-3 in equimolar quantities irrespective of the amounts and the relative proportions of C5, C6, and C7 offered. The amount of C8 bound to EAC1-7 increased with input and at saturation of all C8 binding sites the molar ratio of bound C8/bound C5 approached 1.0. Uptake of C9 by EAC1-8 increased with input and at satura-

[9] Feldman, J. D. 1971. First International Congress of Immunology, Washington, D.C.

tion of all C9 binding sites the molar ratio of bound C9/bound C8 became 6.0. However, calculations suggest that the binding of three C9 molecules to one C8 molecule is sufficient to achieve a full hemolytic effect. Evidence was obtained indicating that binding and hemolytic function of C9 depends upon cooperative interaction of multiple C9 molecules.

Binding of C8 to EAC1-7 and the generation of hemolytic C8 sites were inhibited by antibody to either C5, C6, or C7. Uptake of C9 by EAC1-8 and the generation of hemolytic C9 sites were strongly inhibited by anti-C8 and to a lesser degree by anti-C5. Binding of C9 (but not hemolysis) was also reduced by antibody to C6 or C7.

The data are consistent with the concept that the fully assembled membrane attack mechanism of complement consists of a decamolecular complex: a trimolecular arrangement composed of C5, C6, and C7 forms the binding site for one C8 molecule which in turn furnishes binding sites for six C9 molecules, saturation of three sites apparently being sufficient for expression of full cytolytic activity of the complex. This work made it possible to design a simple molecular model.

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