Articles

Measurement of the Ratio of the Eighth and Ninth Components of Human Complement on Complement-Lysed Membranes†

James L. Stewart, Joseph B. Monahan, Avram Brickner, and James M. Sodetz*

ABSTRACT: The mole ratio of the eighth (C8) and ninth (C9) components of human complement on membranes carrying the cytolytic C5b-9 complex was measured by direct binding assays. Erythrocytes from two different species were used as the membrane system. Antibody-treated sheep erythrocytes carrying a relatively small number of precursive membrane-bound C5b-7 complexes were prepared by exposure to human C8-depleted serum. These complexes were subsequently converted to C5b-8 by addition of saturating amounts of C8. Parallel binding assays using ^{125}I-C8 were used to determine the exact amount bound and thus the number of C5b-8 complexes per cell. These cells were subsequently incubated with excess ^{125}I-C9 and the amount bound relative to C8 on the membrane was measured. Results indicated the C8:C9 ratio remained constant at approximately 1:4 as the number of complexes varied from 40 to 310 per cell. Similar results were obtained regardless of whether C8 and C9 were added sequentially or simultaneously to cells bearing C5b-7. For comparison, experiments were also performed using membranes that contained a high number of complexes. Here, rabbit erythrocytes which carried approximately 25 000 C5b-7 per cell were incubated with limited amounts of C8 to form C5b-8 complexes on the membrane surface, the exact number of which was measured by ^{125}I-C8 binding assays. When erythrocytes prepared in this manner were incubated with excess ^{125}I-C9, the ratio of C8:C9 on the membrane was found to be essentially constant at approximately 1:3 as the number of these complexes varied from 50 to 4000 per cell. These results indicate that under conditions where maximum C9 incorporation would be expected, the C8:C9 mole ratio is approximately 1:3–4 and is independent of the number of cytolytic complexes on the cell membrane.

Complement-mediated cell lysis occurs as a result of the specific association of terminal complement proteins C5b, C6, C7, C8, and C9 to form the membrane-bound cytolytic complex C5b-9 (Müller-Eberhard, 1978). Assembly of C5b-9 is initiated by C5b formation during complement activation and proceeds by a sequential mechanism as follows:

$$\text{C5b} \xrightarrow{\text{C6}} \text{C5b-6} \xrightarrow{\text{C7}} \text{C5b-7} \xrightarrow{\text{C8}} \text{C5b-8} \xrightarrow{n\text{C9}} \text{C5b-9}_n$$

Upon formation of C5b-7, the nascent complex binds to cell membranes through a high-affinity lipid binding site. Once associated with the cell, binding of C8 occurs and the resulting C5b-8 complex incorporates several C9 molecules, yielding C5b-9. This complex alters membrane permeability by a nonenzymatic process resulting in cell lysis.

Properties of C5b-9 studied thus far include its composition, mechanism of assembly, and mode of interaction with the membrane bilayer (Esser, 1982; Bhakdi & Tranum-Jensen, 1983a). Regarding composition, it is generally agreed that C5b-9 contains equimolar amounts of C5b, C6, C7, and C8. However, results in recent years have conflicted with respect to the amount of C9 present in this complex. A value for the C8:C9 molar ratio of approximately 1:3 was obtained by electrophoretic analyses of C5b-9 extracted from complement lysed membranes (Bhakdi et al., 1976; Biesecker et al., 1979). This same value was also obtained from hemolytic titrations using purified C5b-6, C7, C8, and C9 (Podack et al., 1978). In other studies, ratios of 1:6 were observed by directly measuring the binding of C9 to C5b-8 complexes on sheep erythrocytes (Kolb et al., 1972). This ratio was corroborated in later studies of purified C5b-9 which revealed the existence of C9 monomers and disulfide-linked dimers within the complex (Ware & Kolb, 1981). Quantitation of both monomers and dimers by densitometric analyses yielded a C8:C9 ratio of 1:6. Other studies suggested the C8:C9 ratio is dependent on the number of C5b-8 complexes present on the membrane and varies from 1:3 to 1:6 (Kolb & Müller-Eberhard, 1974).

Most recently, indirect binding experiments suggested the C8:C9 ratio may be even less than 1:6 (Podack et al., 1982). The value obtained was reported to be dependent on the number of membrane-bound C5b-8 complexes and varied from 1:6 to 1:16. To explain this variation, it was suggested that C5b-9 consists of a mixture of at least two distinct structures: a monomeric complex designated (C5b-8)$C9_{12-16}$, having a C8:C9 ratio of 1:12–16, and a dimeric structure, $(C5b\text{-}8)_2C9_{12-16}$, with a ratio of 1:6–8. Such heterogeneity is supported by ultrastructural analyses of lysed membranes which indicate that not all the complexes resemble closed circular lesions characteristic of C5b-9 (Tschopp et al., 1982; Tschopp, 1983). This ultrastructural variation has been attributed to factors that presumably limit C9 incorporation, in particular, crowding of C5b-8 complexes or insufficient quantities of C9 present during C5b-9 assembly (Podack et al., 1982; Bhakdi & Tranum-Jensen, 1983b).

In view of this wide range of reported ratios, a more direct method was sought to determine the C8:C9 ratio on complement-lysed membranes. In the present study, we performed binding experiments using erythrocytes bearing membrane-bound C5b-7 and radiolabeled C8 and C9. The C8:C9 ratios were quantitated by measuring the relative amounts of each ligand incorporated during C5b-9 formation. Additionally, we examined the dependency of these ratios on the number of cytolytic complexes on the membrane in order to establish

† From the Department of Chemistry and School of Medicine, University of South Carolina, Columbia, South Carolina 29208. *Received December 21, 1983.* Supported by National Institutes of Health Grant AI-16856 and by Established Investigator Award 82-121 to J.M.S. from the American Heart Association.

0006-2960/84/0423-4016$01.50/0

whether structural heterogeneity could be caused by steric constraints imposed by the number of complexes on the surface.

Experimental Procedures

Purification and Characterization of Proteins. Human C8 was purified as described previously (Steckel et al., 1980). Human C9 was purified from Cohn fraction III by using a procedure similar to one developed by others for plasma (Biesecker & Müller-Eberhard, 1980). Cohn fraction III was kindly provided by Cutter Laboratories. Assays for C8 hemolytic activity were performed as previously described with EAC1-7 cellular intermediates[1] either obtained commercially (Cordis Laboratoris, Miami, FL) or prepared in our laboratory (Steckel et al., 1980; Monahan & Sodetz, 1981). Assays for C9 were performed similarly after converting EAC1-7 to EAC1-8 by addition of saturating amounts of purified C8.

In experiments to determine C8 and C9 molar absorption coefficients, samples of each purified protein were dialyzed into 10 mM sodium phosphate/0.15 M NaCl, pH 7.0, and the absorbance at 280 nm was measured. Parallel samples were dialyzed into 2 mM HCl, and the molar concentration of each protein in this solution was measured by quantitative amino acid analysis performed as described elsewhere (Steckel et al., 1980). Published amino acid compositions and molecular weights were used for C8 (Steckel et al., 1980) and C9 (Biesecker & Müller-Eberhard, 1980). For each sample in buffer and HCl, apparent protein concentrations were then measured by a colorimetric assay and used to relate absorbance at 280 nm to the actual molar concentration determined by amino acid analyses (Schaffner & Weissman, 1973).

Radioiodination of C8 and C9 was performed with either agarose-bound lactoperoxidase or iodogen. The extent of covalent labeling was confirmed by sucrose density gradients and by electrophoretic analysis on polyacrylamide gels in the presence of sodium dodecyl sulfate (Weber & Osborn, 1969). Specific radioactivities were calculated on a mole basis by using the experimentally determined molar absorption coefficient for each protein. Typical values obtained for C8 and C9 were $(4–7) \times 10^{16}$ and $(1–3) \times 10^{16}$ cpm/mol, respectively.

Binding Studies Using Sheep Erythrocyte Intermediates. Sheep blood and hemolysin were purchased from Cordis Laboratories, Miami, FL. Erythrocytes were sensitized to complement activation by treatment with hemolysin to produce EA intermediates which were then converted to EAC1-7 by exposure to human C8-depleted serum as previously described (Monahan & Sodetz, 1981). When necessary, EAC1-7 were further converted to EAC1-8 by incubating with purified C8. Typically, 3 mL of EAC1-7 was suspended at 1×10^9 cells/mL in GIGB and then incubated with 1 mL of C8 prepared at a concentration sufficient for saturation. After incubating at 37 °C for 30 min, the resulting EAC1-8 were centrifuged, washed 3 times with GIGB, and resuspended at 1×10^9 cells/mL for use in C9 binding assays.

Assays to measure the amount of C8 that binds to EAC1-7 were performed in the absence or presence of excess C9. In the absence of C9, assays were performed as described previously with ^{125}I-C8 of known specific radioactivity (Monahan & Sodetz, 1981). Where C8 and C9 were to be present simultaneously, a saturating amount of unlabeled C9 was mixed with increasing amounts of ^{125}I-C8 prior to assay. The assay was then completed as reported previously except that centrifugation was done at 31000*g* to achieve quantitative recovery of lysed membranes.

Binding of C9 was also measured by two different methods. In one, C9 was incubated with EAC1-8 which were preformed as described above. In a typical experiment, 0.3 mL of 1×10^9 EAC1-8/mL was mixed with 0.1 mL of GIGB containing increasing amounts of ^{125}I-C9 and incubated for 35 min at 37 °C. To correct for nonspecific binding, parallel samples were prepared in which EAC1-7 were substituted for EAC1-8. After incubation, intact cells and membranes in each sample were washed 3 times in 0.5 mL of GIGB buffer at 4 °C by repeated centrifugation at 31000*g*. From the known specific radioactivity, the number of moles of C9 bound was calculated. In a control experiment, we confirmed that recovery of intact cells and lysed membranes by centrifugation was quantitative. Here, increasing amounts of unlabeled C9 were added to EAC1-8 containing ^{125}I-C8. Subsequent centrifugation steps consistently recovered >98% of total ^{125}I-C8 bound, regardless of the amount of C9 added.

In the second method, C9 binding was measured in the presence of C8. Here, a saturating amount of unlabeled C8 was mixed with increasing amounts of ^{125}I-C9 in a final volume of 0.1 mL. A 0.3-mL aliquot of EAC1-7 at 1×10^9 cells/mL was added to the mixture and incubated at 37 °C for 35 min. Nonspecific binding was measured by substituting EA for EAC1-7. Washing and quantitation of bound ^{125}I-C9 were performed as described above.

Competition experiments that measured the relative affinity of C8 and ^{125}I-C8 for EAC1-7 were performed as described above for C8 binding assays except that ^{125}I-C8 was held at a constant, saturating level while increasing amounts of unlabeled C8 were added to each incubation mixture. In all cases, unlabeled and labeled ligand was mixed prior to incubating with the cells. The affinity of unlabeled C9 and ^{125}I-C9 for EAC1-8 was measured similarly by holding the ^{125}I-C9 constant and increasing the amount of unlabeled C9 in each incubation mixture. The amount of EAC1-8 and the incubation conditions were as described above for the C9 binding assay.

Binding Studies Using Rabbit Erythrocyte Intermediates. To prepare cells that carried a high density of cytolytic complexes, we utilized the ability of rabbit erythrocytes to activate the alternative complement pathway in human serum (Platts-Mills & Ishizako, 1974). Rabbit erythrocytes were washed and suspended at 2×10^9 cells/mL in 5 mM imidazole/72.7 mM NaCl/0.15 mM $CaCl_2$/0.5 mM $MgCl_2$/2.5% glucose/0.05% gelatin, pH 7.3, and incubated with an equal volume of human C8-depleted serum for 30 min at 37 °C. The resulting EC5b-7 were washed and resuspended in GIGB buffer to 1×10^9 cells/mL. This procedure routinely yielded cells containing ~25 000 C5b-7 sites/cell as measured in ^{125}I-C8 binding assays. This number of sites could be decreased if desired by dilution of the serum.

To prepare EC5b-8 which carried a known amount of C8, 0.3 mL of EC5b-7 was mixed with a limited amount of C8 in 0.1 mL of GIGB buffer and incubated for 30 min at 37 °C. Parallel incubations were performed in which identical molar amounts of ^{125}I-C8 were substituted for C8. In both cases, the resulting EC5b-8 were collected and washed with GIGB

[1] Abbreviations: EA, antibody-treated sheep erythrocytes; E, rabbit erythrocytes; EAC1-7, EAC1-8, and EAC1-9, EA carrying bound complement proteins C1 through C7, C1 through C8, and C1 through C9, respectively; EC5b-7, EC5b-8, and EC5b-9, E carrying the intermediate complement complexes C5b-7, C5b-8, and C5b-9, respectively; GIGB, buffer containing 5 mM imidazole, 72.7 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose, 0.05% gelatin, and 5 mg/mL bovine serum albumin, pH 7.3 at 4 °C; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Complement proteins are named in accordance to *Bull. W.H.O.* (1968).

buffer by repeated centrifugation at 31000*g*. The exact mole quantity of C8 bound was determined from samples containing ^{125}I-C8 while the corresponding samples containing unlabeled C8 were used in C9 binding experiments. Nonspecific binding in these assays was measured by substituting E for EC5b-7. It is noted that high centrifugation speeds were required because we observed that large amounts of C8 bound to EC5b-8 caused significant lysis. Thus, recovery of both intact EC5b-8 and lysed EC5b-8 membranes was necessary to obtain an accurate measurement of bound ^{125}I-C8.

Binding of ^{125}I-C9 to EC5b-8 was measured by assays similar to those described for EAC1-8. The EC5b-8 used here carried a known mole quantity of C8 as determined above. Although higher numbers of C5b-8 sites/cell produced a mixture of intact EC5b-8 and lysed EC5b-8 membranes, this mixture was used in C9 binding studies since control experiments indicated that membrane-bound C5b-8 was uniformly distributed between intact and lysed EC5b-8. In a typical binding experiment, 0.3 mL of EC5b-7 at 1×10^9 cells/mL in GIGB buffer was incubated with sufficient C8 to yield the desired amount bound. Both intact and lysed EC5b-8 were then quantitatively recovered by centrifugation at 31000*g*, washed, and resuspended in 0.3 mL of GIGB. Varying amounts of ^{125}I-C9 in 0.1 mL of GIGB were added and incubated for 35 min at 37 °C. After washing, binding was quantitated as described for the sheep systems. Nonspecific binding was measured by substituting EC5b-7 for EC5b-8. It is noted that centrifugation steps employed in this binding procedure recovered >97% of intact and/or lysed EC5b-9 when measured in control experiments.

Results

Characterization of ^{125}I-C8 and ^{125}I-C9. To ensure the validity of the approach used in this study, it was necessary to establish several facts about ^{125}I-C8 and ^{125}I-C9. The first was that radiolabeling itself had no significant effect on the activity of these proteins. Regarding this, we compared the hemolytic activity of the radiolabeled proteins to the activity of their unmodified precursors and found no significant difference. For purified C8, the specific hemolytic activity was typically $(3.9 \pm 0.4) \times 10^7$ serum CH50 units/mg and remained at 88–98% of this level after labeling. For purified C9, the specific activity was $(3.3 \pm 0.4) \times 10^7$ serum CH50 units/mg, and 85–95% of this activity was retained after radiolabeling. Second, it was necessary to demonstrate that all of the radiolabel was covalently bound to C8 and C9. This was established by quantitative SDS–PAGE analyses which indicated >95% of the applied radioactivity migrated with the C8 subunits or C9. Third, it was important to determine that labeling did not induce significant changes in the physical state of these proteins. This was of particular concern since recent reports indicated that C9 is capable of spontaneous self-polymerization (Podack & Tschopp, 1982). We examined ^{125}I-C8 and ^{125}I-C9 on sucrose density gradients and found both to be monomeric with no evidence of any aggregation caused by labeling. Lastly, if quantitative binding studies were to be used to measure C8:C9 ratios on lysed membranes, accurate molar specific radioactivities for ^{125}I-C8 and ^{125}I-C9 were needed. This necessitated the measurement of extinction coefficients for these proteins which was accomplished by procedures described above. Values of $\epsilon_{280}^{1\%} = 14.9$ and 9.88 were determined for C8 and C9, respectively, while the corresponding molar extinction coefficients were calculated to be $\epsilon = 2.25 \times 10^5$ and 7.10×10^4 M^{-1} cm^{-1}.

Ratio of C8:C9 on Membranes Bearing a Low Number of Cytolytic Complexes. Having established that C8 and C9 can be radioiodinated without significant alteration of their properties, we next utilized these derivatives in direct binding experiments to determine their stoichiometric ratio on complement-lysed membranes. Initial experiments used sheep erythrocytes as the membrane source since they can be prepared so as to bear relatively few C5b-7 sites per cell.

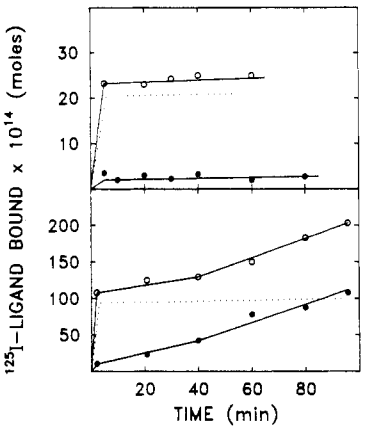

FIGURE 1: Time dependence of C8 and C9 binding to erythrocyte intermediates. (Top) A saturating amount of ^{125}I-C8 in 0.1 mL of GIGB was incubated at 37 °C with 0.3 mL of either EA (●) or EAC1-7 (○) prepared at 1×10^9 cells/mL in the same buffer. At the indicated times, cells were centrifuged at 300*g* and washed, and the amount of ^{125}I-C8 bound was quantitated. The dotted line is the result obtained after nonspecific binding to EA is subtracted. (Bottom) Binding of ^{125}I-C9 to EAC1-7 (●) or EAC1-8 (○) was measured under the same conditions as above except that samples were centrifuged at 31000*g* to recover both intact cells and lysed membranes. The dotted line is the result after nonspecific binding to EAC1-7 is subtracted.

In the first control experiment, we examined the time dependency of C8 and C9 binding to ensure that incubation times were sufficient to permit maximum incorporation of each ligand. For C8, the experiment was performed by incubating excess ^{125}I-C8 with EAC1-7 and measuring the amount bound as a function of time. The results in Figure 1 show that specific binding of C8 occurs within minutes and does not increase with further incubation. In the corresponding experiment which measures the time dependency of ^{125}I-C9 binding to EAC1-8, results indicate that binding again is rapid and saturation is reached within minutes. With further incubation, a gradual increase in ^{125}I-C9 binding is observed, but it is nonspecific in nature as evidenced by a similar increase in binding to EAC1-7. This behavior was of interest in view of the reports on the polymerization of C9 and its ability to bind to lipid vesicles. The increased nonspecific binding was thought to be attributed to such spontaneous aggregation of C9 in the incubation mixture and subsequent binding to membranes. However, when the supernatant from the 100-min incubation mixture was analyzed on sucrose gradients, we found that ^{125}I-C9 was still monomeric, thus excluding aggregation as the cause of increased nonspecific binding during prolonged incubation. By use of data in Figure 1, optimum incubation times of 30 and 35 min were chosen for C8 and C9 binding, respectively. At these times, specific binding of each ligand is complete while nonspecific binding is minimal.

In a second control experiment shown in Figure 2, the binding of ^{125}I-C8 and ^{125}I-C9 was compared to that of unlabeled C8 and C9. This experiment was designed to establish whether radiolabeling had any effect on the affinity of these proteins for their respective binding sites on EAC1-7 and EAC1-8. Importantly, results show that for both ^{125}I-C8 and ^{125}I-C9, ~50% inhibition of binding occurs when an equimolar amount of unlabeled ligand is added to the mixture. Such competition indicates the labeled and unlabeled proteins have

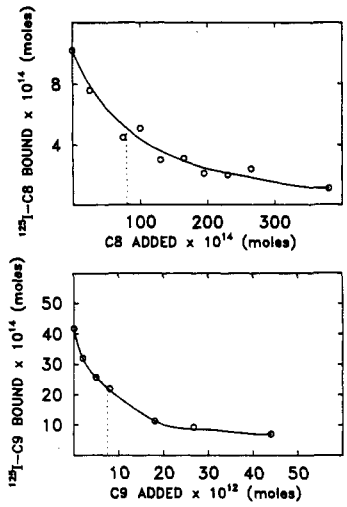

FIGURE 2: Comparison of unlabeled and labeled C8 and C9 binding to erythrocyte intermediates. (Top) Competition between C8 and ^{125}I-C8 for binding to EAC1-7. Incubation mixtures containing EA or EAC1-7 were prepared as in Figure 1 but contained increasing amounts of unlabeled C8 along with a fixed, saturating amount of ^{125}I-C8 (8×10^{-13} mol). Incubations were performed for 30 min at 37 °C. Bound ^{125}I-C8 was quantitated as described in Figure 1 and was corrected by subtracting nonspecific binding to EA controls. The dotted line indicates the amount of unlabeled C8 required to achieve 50% inhibition of ^{125}I-C8 binding. (Bottom) Competition between C9 and ^{125}I-C9 for binding to EAC1-8. Incubation mixtures prepared as in Figure 1 consisted of EAC1-7 or EAC1-8 and increasing amounts of unlabeled C9 along with a fixed, saturating amount of ^{125}I-C9 (8×10^{-12} mol). Incubations were performed for 35 min at 37 °C. Bound ^{125}I-C9 was quantitated as in Figure 1 and corrected for nonspecific binding to EAC1-7 controls. The dotted line indicates the amount of unlabeled C9 required to inhibit 50% of the ^{125}I-C9 binding.

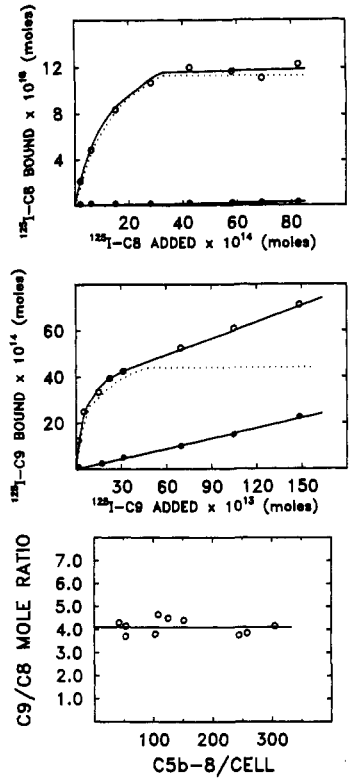

FIGURE 3: Binding of C8 and C9 upon sequential addition to sheep erythrocyte intermediates. (Top) Shown are typical binding results obtained when increasing amounts of ^{125}I-C8 are added to 0.3×10^9 EA (●) or EAC1-7 (○). Samples of ^{125}I-C8 were incubated with the cells for 30 min at 37 °C as described in Figure 1. The dotted line shows the amount of ^{125}I-C8 bound after nonspecific binding to EA is subtracted. (Middle) Binding curves obtained when increasing amounts of ^{125}I-C9 are added to 0.3×10^9 EAC1-7 (●) or EAC1-8 (○). The EAC1-8 used here were prepared exactly as above but contained a saturating amount of unlabeled C8. Mixtures of ^{125}I-C9 and each cell type were incubated for 35 min at 37 °C as described in Figure 1. The dotted line shows the binding of ^{125}I-C9 after subtracting nonspecific binding to EAC1-7. (Bottom) Relationship of C8:C9 ratio to the number of cytolytic complexes. Experiments were performed as above with EAC1-7 bearing different amounts of C5b-7. The C8:C9 ratios obtained are expressed as a function of the number of C5b-8 complexes which could be formed on these cells by addition of saturating amounts of C8.

similar affinities, and thus radiolabeling does not significantly alter their binding properties.

We next measured the ratio of C8:C9 bound when each are added sequentially to EAC1-7. Typical results from assays used to measure the amount of C8 bound are shown in the upper panel of Figure 3. Results here are similar to those reported previously and show that ^{125}I-C8 binds to EAC1-7 with high affinity, binding is saturable, and nonspecific binding as measured by using EA is negligible. For this particular EAC1-7 preparation, the number of C8 molecules bound and thus the number of C5b-8 sites formed at saturation are ~220 per cell. In a corresponding experiment, substitution of saturating amounts of unlabeled C8 for ^{125}I-C8 was made in the incubation with EAC1-7 to yield EAC1-8 which carried unlabeled protein. These cells were then used in binding assays to determine the amount of ^{125}I-C9 bound at saturation. Results from these experiments are shown in the middle panel of Figure 3. It is noted that binding of ^{125}I-C9 to EAC1-8 increases rapidly at first but then only gradually as higher amounts of ligand are added. This gradual increase at high ^{125}I-C9 levels is attributed to nonspecific interactions since a parallel increase in binding to EAC1-7 controls is also observed. It is noted that nonspecific binding is negligible at low levels of ^{125}I-C9 added and represents ~10% of total ^{125}I-C9 bound near the point of saturation. When corrected, results show that saturation of specific binding sites occurs when the number of C9 molecules bound is ~880 per cell. From results in Figure 3, it is concluded the C8:C9 ratio on these particular lysed membranes is ~1:4.

Results in the lower panel of Figure 3 summarize the C8:C9 ratios obtained when experiments such as those above are performed on sheep erythrocytes bearing different amounts of C5b-7. Significantly, little variation in the C8:C9 ratio occurs over an 8-fold range in the number of cytolytic complexes. Further, extrapolation of the data to one complex per cell yields a C8:C9 ratio of 1:4, a value comparable to that observed at 310 per cell.

For comparative reasons, we also performed measurements of C8:C9 ratios using modified assays in which both ligands were present *simultaneously* during binding, though only one carried radiolabel. To measure C8 binding, increasing amounts of ^{125}I-C8 were mixed with a saturating amount of unlabeled C9 and then added to EAC1-7 cells. Conversely, to measure C9 binding in these modified assays, increasing amounts of ^{125}I-C9 were mixed with saturating amounts of unlabeled C8 prior to incubation with cells. Representative results of these assays are shown in Figure 4. For the EAC1-7 preparation used in this particular experiment, ~340 molecules of C8 and ~1500 molecules of C9 are bound per cell at saturation to yield a C8:C9 ratio of 1:4.4. Figure 4 also summarizes results from similar experiments with cells bearing different amounts of C5b-7 and thus yielding varying numbers of cytolytic complexes. As was the case when C8 and C9 were added sequentially to EAC1-7 (Figure 3), the C8:C9 ratio remains essentially constant albeit the extrapolated value is slightly higher at ~1:4.6. These results indicate that se-

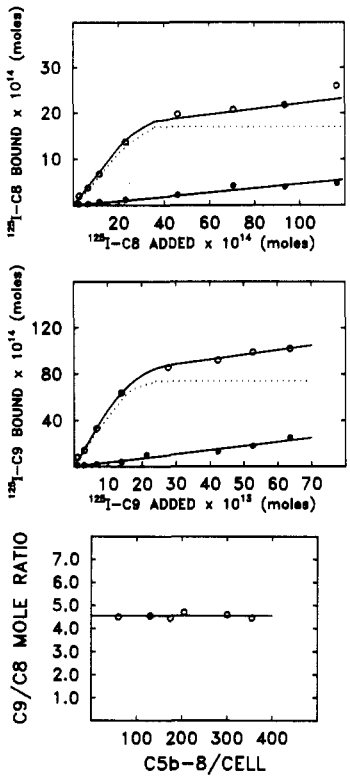

FIGURE 4: Binding of C8 and C9 upon simultaneous addition to sheep erythrocyte intermediates. (Top) Increasing amounts of ^{125}I-C8 and a constant, saturating amount of unlabeled C9 contained in 0.1 mL GIGB were incubated as described in Figure 1 with 0.3×10^9 EA (●) or EAC1-7 (○). Incubations were performed for 35 min at 37 °C. The dotted line shows results after correcting for nonspecific binding to EA. (Middle) Increasing amounts of ^{125}I-C9 were mixed with a saturating amount of unlabeled C8 and incubated as above with 0.3×10^9 EA (●) or EAC1-7 (○). The dotted line has been corrected for nonspecific binding. (Bottom) Relationship of C8:C9 ratio to the number of cytolytic complexes. Experiments were performed as above with EAC1-7 bearing different amounts of C5b-7. Measured C8:C9 ratios are expressed relative to the number of C5b-8 complexes which could be formed on these cells upon addition of saturating amounts of C8.

quential or simultaneous addition of C8 and C9 to EAC1-7 makes little difference in the amount bound at saturation.

Ratio of C8:C9 on Membranes Containing a High Number of Cytolytic Complexes. To further investigate the dependency of C8:C9 ratios on the number of cytolytic complexes, we next performed binding experiments using rabbit erythrocytes. This system was chosen because of the relatively high number of C5b-7 complexes that assemble on the membrane as a consequence of alternative pathway activation. Rabbit erythrocytes exposed to C8-depleted human serum typically carried ~25 000 C5b-7 complexes per cell as measured in ^{125}I-C8 binding assays. One potential problem noted with these cells was that when converted to EC5b-8, a significant amount of lysis occurs, which varies with the amount of C8 added. This C8-mediated hemolysis was not surprising considering the large number of C5b-8 complexes being generated, but it did raise a concern that, in the presence of a mixture of intact cells and lysed membranes bearing C5b-7, C8 may preferentially bind to one over the other. Consequently, we performed control experiments shown in Figure 5 to determine the eventual distribution of C5b-8 complexes after C8 is added to EC5b-7. The results indicate that, with increasing amounts of ^{125}I-C8 added, hemolysis increases but ^{125}I-C8 becomes uniformly distributed between intact cells and lysed membranes. This confirms that C8 is not discriminating between C5b-7 on the remaining whole cells and lysed membranes.

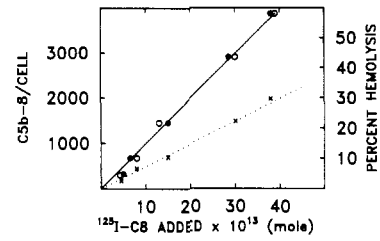

FIGURE 5: Distribution of C8 between intact and lysed rabbit EC5b-8. Duplicate samples of EC5b-7 were incubated with increasing amounts of ^{125}I-C8 as described in the text. One sample was centrifuged at 1000*g* to collect intact EC5b-8. The number of intact cells recovered and the corresponding amount of bound ^{125}I-C8 were used to calculate the number of C5b-8 complexes formed per cell (○). A second sample was centrifuged at 31000*g* to determine the amount of ^{125}I-C8 bound to both intact and lysed EC5b-8. By use of this value, the number of C5b-8 complexes was calculated on the basis of the number of intact cells initially present in the incubation mixture (●). Hemolysis occurring as a result of adding C8 to EC5b-7 was determined from the absorbance at 541 nm of the 31000*g* supernatants (×).

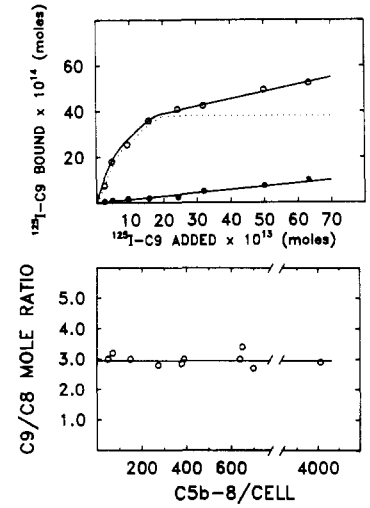

FIGURE 6: Binding of C8 and C9 to rabbit erythrocyte intermediates. (Top) Quantitation of C9 binding to rabbit EC5b-8. Shown are typical binding results obtained when increasing amounts of ^{125}I-C9 are incubated with 0.3×10^9 EC5b-7 (●) or EC5b-8 (○). The EC5b-7 used in this particular experiment carried ~25 000 C5b-7 complexes per cell but were incubated with a limited amount of C8 to yield only 280 C5b-8 per cell. Binding of ^{125}I-C9 to these complexes was quantitated as described in the text. The dotted line shows results obtained after subtracting nonspecific binding to EC5b-7 controls. Approximately 4×10^{-13} mol of ^{125}I-C9 is bound at saturation, which corresponds to ~800 C9 molecules per cell. (Bottom) Relationship of C8:C9 ratio to the number of cytolytic complexes on rabbit erythrocytes. Experiments identical with those above were performed using erythrocytes bearing different numbers of C5b-8. In each case, the C8:C9 ratios were measured and are expressed as a function of the number of C5b-8 on the cell.

We next established conditions to measure C9 binding to EC5b-8. Results from a representative experiment are shown in the upper panel of Figure 6. The EC5b-8 used here carry a known quantity of C8 as determined independently from ^{125}I-C8 binding assays. As with the sheep system, the incubation time was determined to be sufficient for maximum C9 binding. As increasing amounts of ^{125}I-C9 are added, saturation is achieved at a point where the C8:C9 ratio is ~1:3. Binding is specific as evidenced by negligible ^{125}I-C9 binding to EC5b-7. When a series of similar experiments were performed in which EC5b-8 bearing varying numbers of C5b-8 complexes per cell were used, the results shown in the lower panel of Figure 6 were obtained. The C8:C9 mole ratio is seen to be essentially constant as the number of complexes increases from 50 to 4000 per cell. Extrapolation of the data to one per

cell yields a C8:C9 ratio of ~1:3.

In addition to experiments described in Figure 6, we also prepared EC5b-7 bearing fewer C5b-7 by diluting the human C8-depleted serum used in their preparation. The purpose of these experiments was to confirm results obtained when each cell preparation contained ~25 000 C5b-7 complexes per cell, only a portion of which were converted to C5b-8. In these latter experiments, we prepared EC5b-7 containing only 360 and 760 C5b-7 per cell. These were incubated with saturating amounts of C8 to completely convert them to C5b-8. When used in ^{125}I-C9 binding studies as described above, these cells yielded similar C8:C9 ratios of 1:3.1 and 1:3.2, respectively.

Discussion

Results in this study indicate the ratio of C8:C9 on membranes subjected to complement-mediated lysis under a variety of conditions is 1:3–4. Similar results were obtained by using two different erythrocyte membrane systems and two different mechanisms of complement activation. Within a broad range, this ratio exhibited no dependency on the number of cytolytic complexes formed on the membrane and remained essentially unchanged when extrapolated to one complex per cell. Furthermore, the same ratio was obtained regardless of whether the cytolytic complex was formed by adding C8 and C9 sequentially or simultaneously to the precursive C5b-7 complex.

Our experimental method for measuring this ratio has distinct advantages over several of those used previously. Most important is the fact that measurements are made in situ rather than after extraction and purification of C5b-9, a procedure which could significantly alter its composition. A related advantage is that C8:C9 ratios can be accurately quantitated since specific incorporation of ^{125}I-C8 and ^{125}I-C9 is measured directly. This is preferable to methods based on electrophoresis and densitometric analyses of C5b-9 constituents which are qualitatively useful but quantitatively less reliable. Because our approach measures binding of C8 and C9 directly, saturation is also more clearly defined than in those methods based on dose–response phenomena such as hemolytic titrations or marker release. Essential to the validity of our approach, however, is the requirement that radiolabeling not significantly alter the properties of C8 and C9. This was found to be the case as judged by several criteria, the most significant being the similar binding affinities of the unlabeled and labeled proteins as demonstrated by competition assays.

Our results agree with those from earlier studies which used indirect methods and measured a C8:C9 ratio of 1:3. Our results also agree with a more recent study in which direct binding experiments were performed using purified terminal components and resealed human erythrocyte ghosts (Sims, 1983). The aim of that study, however, was distinct from ours in that it attempted to correlate the assembly of C5b-9 with the onset of functional membrane damage. To do this, the binding of radiolabeled C8 and C9 to ghosts bearing C5b-7 was measured relative to the production of sucrose-permeant membrane pores. Significantly, the C8:C9 ratio did not vary with the extent of membrane damage and remained constant at 1:3 from an extrapolated value of one cytolytic complex per cell to ~1000 per cell. These results are consistent with our observation that the C8:C9 ratio does not vary with the number of complexes on the cell.

Results in the present study differ from those that concluded the C8:C9 ratio is less than 1:6. One recent study in particular reported the ratio varied from 1:6 to 1:16 and was dependent on the number of cytolytic complexes formed as well as the input of C9 (Podack et al., 1982). This conclusion was based on indirect binding experiments in which trace amounts of radiolabeled C8 or C9 were mixed with increasing amounts of unlabeled precursor prior to incubating with rabbit erythrocytes bearing C5b-7 or C5b-8, respectively. Ratios were measured at saturation, which was defined as the amount of unlabeled ligand which could be added before binding of the trace amount of labeled ligand was inhibited. Importantly, this approach assumes that, prior to saturation, *all* the unlabeled ligand added to the system will bind. The validity of this assumption is questionable since our binding results indicate that at any given level, only a portion of added ligand binds. For example, data in Figure 3 show that only ~30% of added ^{125}I-C8 or ~10% of added ^{125}I-C9 is bound at a point near saturation. This need for excess ligand to achieve a particular level of binding is not an artifact caused by radiolabeling since results in Figure 2 show unequivocally that the unlabeled and labeled proteins have comparable affinities for their respective binding sites. Rather, it likely reflects the intrinsic affinity each has for its precursive complex. If in the above-mentioned study it was recognized that binding was not complete, and if differences between C8 and C9 affinities were accounted for, the ratios obtained would be significantly different and would more closely approximate values measured in our study.

Our results have important ramifications with regard to understanding the structure of C5b-9 as it actually exists on the lysed membrane. It has been proposed that within this complex, C9 forms a dodecameric ringlike structure which is associated with one or possibly two C5b-8 complexes. This conclusion is based largely on ratios measured in the indirect binding study cited above and on ultrastructural similarities between C5b-9 and the structure formed on lipid vesicles as a result of heat-induced polymerization of C9. Other ultrastructure evidence suggests that heterogeneity of C5b-9 structure may be more extensive in that not all the complexes form closed circular lesions. If indeed such structural diversity exists, this raises the question as to what factors contribute to this heterogeneity.

Our results argue against several possibilities. One proposed previously is insufficient or limiting amounts of C9, particularly when C5b-9 is formed directly from serum. In our study, this could not be a contributing factor since in all our experiments, C5b-9 was formed in the presence of saturating amounts of exogenous C9. Another factor which reportedly can limit the amount of C9 bound and thus increase the C8:C9 ratio is the number of C5b-9 complexes formed on the membrane. In our study, steric constraints caused by the spatial arrangement of complexes are not likely responsible for high C8:C9 ratios. For the sheep system, results from cells bearing as few as 36 C5b-9 complexes per cell, a condition where steric constraints should be minimal, were similar to those for cells bearing as many as 350. Furthermore, similar ratios were obtained for rabbit erythrocytes bearing 25 000 C5b-7 complexes per cell of which a portion were converted to C5b-9. Here, concern over steric factors is particularly justified in view of the high number of C5b-7 yet the same results were obtained by using EC5b-7 bearing relatively few C5b-7 per cell. With regard to C5b-7, it might be argued that clustering of this complex could occur as it is formed on the membrane of either cell system. If such clustering of this precursor was extensive, it may sterically inhibit subsequent C5b-9 assembly and thus affect the C8:C9 ratio. While such a possibility cannot be excluded, it seems unlikely considering the constant ratio observed over a range of 36 to 25 000 C5b-7 per cell.

Another possibility that would result in heterogeneity and influence the C8:C9 ratio is if clustering or aggregation of

C5b-8 complexes on the membrane were necessary for maximum C9 binding. If so, this would mean that, in any population of membrane-bound C5b-8, some complexes might exist as monomers unable to bind C9 and some as multimers capable of interacting with C9. Accordingly, the observed C8:C9 ratio would be high. An absolute requirement for clustering seems unlikely in view of our data because if it were essential, one would expect the C8:C9 ratio to vary with the number of C5b-8 complexes on the membrane surface. This does not occur as is particularly evident from results using rabbit erythrocytes shown in Figure 6 where the lowest number of C5b-8 used was 50 per cell. These cells were prepared by binding 50 C8 molecules to erythrocytes containing 25 000 C5b-7 per cell. Considering the number of C5b-7 available for binding, it seems unlikely this small quantity of added C8 would form clusters of C5b-8 complexes. Yet, the C8:C9 ratio is 1:3, a value identical with that observed at 4000 C5b-8 per cell.

A high C8:C9 ratio might also be expected if a significant percentage of C5b-8 sites decayed spontaneously after formation and thus were unable to bind C9. Decay of both C5b-7 and C5b-8 sites on erythrocytes during prolonged incubation at 37 °C was suggested by others in a preliminary report (Lint et al., 1982). In our experiments, we observed no decrease in C9 binding capacities of either EAC1-8 or EC5b-8 during the incubation period used in our assays. This was confirmed in control experiments which showed that cells prepared by incubating EAC1-7 or EC5b-7 with C8 for 15 or 45 min at 37 °C yielded the same C8:C9 ratio when subsequently exposed to saturating amounts of C9. Hence, the number of functional C5b-8 per cell remains constant during this incubation period. Further evidence that decay is unlikely is provided by results obtained when C8 and C9 are added simultaneously to EAC1-7 (Figure 4). Here, C9 can bind immediately upon formation of C5b-8 yet the C8:C9 ratio is the same as when C9 is added to the preformed complex on EAC1-8 (Figure 3).

The C8:C9 ratio measured in our experiments must still be reconciled with the possibility that it is a dodecamer of C9 which forms the circular structure associated with the cytolytic lesion. Our ratio would be compatible with such a structure only if it were assumed that 3–4 C5b-8 complexes are associated with the C9 polymer. This implies that clustering of C5b-8 is required for binding. Our results argue against this in that no dependency of C8:C9 ratios on the number of C5b-8 complexes was observed. Furthermore, recent ultrastructural studies have provided direct evidence of only one C5b-8 complex at a typical lesion site (Tschopp et al., 1982). An alternative explanation for our results is that monomeric C5b-8 complexes incorporate varying amounts of C9 and thus yield an average C8:C9 ratio of 1:3–4. Some of these would incorporate amounts sufficient to form the circular structure of a cytolytic lesion and others would incorporate less or none. This explanation is not consistent, however, with the fact that when measurements are extrapolated, the same ratio is observed for only one complex per cell. It should be noted though that such an extrapolation is meaningful only if C5b-8 and C9 interaction is noncooperative at a very low number of complexes per cell.

On the basis of our results alone, we cannot confirm nor eliminate structural heterogeneity as a factor in our observed ratio. Consequently, we can neither support nor refute the existence of a dodecameric C9 structure within C5b-9. However, our study has demonstrated that heterogeneity, if it exists, is not directly attributable to factors previously thought to be of primary importance, in particular, limiting amounts of C9, steric constraints imposed by the number of C5b-9 complexes, and inherent lability of the C5b-8 intermediate.

Registry No. C8, 80295-58-5; C9, 80295-59-6; C5b-9, 82986-89-8.

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