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COMPLEMENT PORES IN ERYTHROCYTE MEMBRANES

ANALYSIS OF C8/C9 BINDING REQUIRED FOR FUNCTIONAL MEMBRANE DAMAGE

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The number of membrane-bound terminal complement proteins (C5b-9) required to generate a functional pore in the human erythrocyte membrane ghost has been determined. Resealed erythrocyte ghost membranes (ghosts) were treated with human complement proteins C5b6, C7, ^{131}I-C8, and ^{125}I-C9 under non-lytic conditions. Following C5b-9 assembly, sucrose-permeant ghosts were separated from C5b-9 ghosts that remained impermeant to sucrose by centrifugation over density barriers formed of 43% (w/v) sucrose. Analysis of ^{131}I-C8 and ^{125}I-C9 bound to sucrose-permeant and sucrose-impermeant subpopulations of C5b-9 ghosts revealed: 1. Sucrose-permeant C5b-9 ghosts show increased uptake of both ^{131}I-C8 and ^{125}I-C9 as compared to ghosts that remain impermeant to sucrose. Ghosts with less than 300 molecules ^{131}I-C8 bound remain impermeant to sucrose, irrespective of the total C9 input, or, the multiplicity of C9 uptake by membrane C5b-8. 2. In the presence of excess ^{125}I-C9, the ratio of ^{125}I-C9/^{131}I-C8 bound to membrane C5b67 is 3.2 ± 0.8 (mean ±2 S.D.), suggesting an average stoichiometry of 3 C9 per C5b-8. Under these conditions, the ratio of ^{125}I-C9/^{131}I-C8 bound to sucrose-permeant ghosts (3.3 ± 0.7) does not significantly differ from the ratio bound to sucrose-impermeant ghosts (2.9 ± 0.6). 3. With limiting C9 input, the threshold of total C5b-8 uptake required for sucrose permeability increases significantly above 300 per cell when the ratio of bound ^{125}I-C9/^{131}I-C8 is decreased below unity. In the complete absence of C9, 11700 C5b-8 complexes are bound to sucrose-permeant ghosts. It is concluded that more than 300 C5b-9 complexes must bind to the human erythrocyte to form a sucrose-permeant lesion. Although the binding of one C9 per C5b-8 is critical to the pore-forming activity of these proteins, the binding of additional molecules of C9 to each complex (C9/C8 > 1) does not significantly alter the threshold of total C5b-9 uptake required for lesion formation.

Introduction

The cytolytic activity of the complement system resides in the five proteins C5b, C6, C7, C8 and C9 [1]. By spontaneous assembly into macromolecular complexes (C5b-9), these serum proteins expose hydrophobic domains that insert into membranes, thereby increasing permeability to aqueous solute [2–5]. When bound to the plasma membrane of a cell, increased transmembrane ion flow initiated by these proteins can collapse electrochemical gradients and result in its lytic rupture [6–8].

Two general models have been considered to account for the membrane permeability changes initiated by C5b-9 (reviewed in Ref. 9). As de-

Abbreviations: C, complement proteins are named in accordance with recommendations cited in Ref. 43; ghosts, resealed human erythrocyte membrane ghosts.

picted by the 'doughnut hole' model, the proteins of each C5b-9 complex encompass a large aqueous pore inserted through the lipid bilayer, creating a transmembrane channel across which ions diffuse [2,8,10]. According to the 'leaky patch' model, the permeability-promoting properties of the C5b-9 proteins derive from their capacity to bind and disorder membrane lipid, generating extended domains of non-bilayer structure across which ions diffuse [4,11]. The doughnut hole model is suggested by the one-hit kinetics observed for complement hemolysis, and by the ultrastructural features of the assembled proteins, depicted by electron microscopy as forming uniform, annulus-ringed hollow cylinders embedded into membrane lipid [2,12–16]. The leaky patch model is suggested by the capacity of the C5b-9 proteins to significantly decrease the anisotropy of ordered lipid bilayers, and to cause lytic breakdown of membranes in the absence of osmotic gradients [11,17].

Recently, it has been reported by several laboratories that the effective solute exclusion radius of the membrane lesions initiated by the complement proteins can vary considerably, depending upon the relative input of the five proteins composing the C5b-9 complex [18–25]. This observation has led to various proposals for how the functional properties of the membrane lesion relate to the stoichiometry and/or aggregation state of membrane-bound C5b-9 complexes. Based upon the known multiplicity of C9 binding to C5b-8 [26,27] – and the increased effective pore radius measured with increased C9 input to C5b-8 cells [18,19] – Boyle et al. [9] proposed that the size of the complement pore is related to the stoichiometry of C9. According to their model, each pore in the membrane is formed by a single C5b-9 complex, its size determined by the number of molecules of C9 that bind to C5b-8. A similar model has recently been proposed by Ramm et al. [23]. Alternatively, Sims and Lauf [21] and Sims [24] proposed that multiple C5b-9 complexes which are bound to the same membrane interact to generate a lesion that expands as the density of C5b-9 complexes is increased. This proposal was based on the observation that the average size of the membrane lesion increases under all conditions favoring increased C5b-9 assembly, irrespective of the ratio of the input of C9 to C5b-8 [21,23]. Finally, analysis by Delisi et al., [25] suggested that both the multiplicity of C5b-9 binding – as well as the C9 to C5b-8 ratio of the assembled complexes – contribute to the functional properties of these proteins.

In each of these previous studies, information pertaining to the total number and stoichiometry of membrane-bound C5b-9 complexes contributing to the membrane lesion was derived from an analysis of how C5b-9 pore function related to the concentration of the C5b-9 proteins to which target cells were exposed (protein input commonly expressed in terms of a relative hemolytic titer). It was of interest, therefore, to also determine how the onset of C5b-9 pore function relates to the quantitative membrane binding of the C5b-9 proteins per se. Accordingly, in the present study, resealed human erythrocyte ghost membranes were exposed to the purified C5b-9 proteins and then separated on the basis of the exclusion size of the resulting membrane pore by floatation on a density barrier of sucrose. The quantitative incorporation of radiolabeled C8 and C9 into C5b-9 complexes bound to the sucrose-permeant and sucrose-impermeant sub-populations of cells has then been compared, in order to determine directly whether the formation of a functional lesion in these membranes is related to (i) the total multiplicity of membrane uptake of C5b-9 complexes, (ii) the C9 to C5b-8 ratio of the membrane-bound proteins.

Materials and Methods

Solutions

All solutions were freshly prepared using H_2O obtained by reverse osmosis and ultrafiltration (Millipore, Bedford, MA). Except where indicated otherwise, all buffers contained 0.02% (w/v) NaN_3. KCl-Tris: 165 mM KCl/2 mM Tris, pH 7.20. KCl-Tris-albumin: KCl-Tris made 0.5% (w/v) in fatty acid-free bovine albumin (Sigma, St. Louis, MO). Sucrose cushion: 43% (w/v) sucrose in 50 mM KCl/25 mM Tris, pH 7.20. Phosphate-buffered saline: 150 mM NaCl/10 mM sodium phosphate, pH 7.20.

Radiochemicals. $Na^{125}I$ and $Na^{131}I$ were obtained in dilute NaOH solution from Amersham International (Chicago, IL). [^{14}C]sucrose (sucrose

[U-^{14}C]), M_r 342.3, spec. act. 396 mCi/mmol) was obtained from New England Nuclear (Boston, MA).

Complement proteins

Complement proteins C5b-6 [29] and C7 [30] were prepared from freshly-drawn human serum according to published procedures. The IgG fraction of goat monospecific antiserum to C7 required for these procedures was a generous gift of Dr. Alfred F. Esser (Gainesville, FL). C8 [31] and C9 [32,33] were isolated from Cohn Fraction III of human serum according to published procedures. Cohn Fraction III was a generous gift of Mr. Lewis Larson (MA Public Health Biological Laboratories, Boston). All purified proteins were stored at concentrations of 1 mg/ml either at −85°C, or, at −20°C in the presence of 40% (v/v) glycerol. Immediately prior to radiolabeling, C8 and C9 were each rechromatographed (0.9 × 60 cm bed) on Sephacryl S200 (Pharmacia, Piscataway, NJ) in azide-free phosphate-buffered saline to remove aggregates formed during storage. The concentrations of these proteins were determined by the absorbance at 280 nm, using extinction coefficients of $\epsilon = 1.6$ and $\epsilon = 0.96$ ml · mg^{-1} · cm^{-1} for C8 and C9, respectively [34]. Molecular weights of 151000 [31] and 71000 [32] for C8 and C9, respectively, were used to determine molar quantities of the proteins.

Hemolytic assays

The functional activity of the purified complement proteins (and radiolabeled derivatives, see below) prepared for these studies were assessed by hemolytic titrations using the sensitized erythrocyte intermediate EAClgp4-7hu and functionally-pure human C8 (100 CH50/ml) and C9 (100 CH50/ml) obtained from Cordis Laboratories (Miami, FL). Pooled sera obtained from healthy adults and stored aliquoted at −85°C served as a reference control. The hemolytic activity (CH50) of the reference serum tested against $2 \cdot 10^7$ erythrocytes in the Cordis Assay was typically $1.4 \cdot 10^5$ CH50/ml for C8 and $5.6 \cdot 10^4$ CH50/ml for C9. Based upon reported [9] concentrations of these proteins in normal serum of 55 μg/ml (C8) and 60 μg/ml (C9), the apparent specific activities of these proteins in the reference serum was determined to be $2.5 \cdot 10^6$ CH50/mg and $9.3 \cdot 10^5$ CH50/mg for C8 and C9, respectively. By comparison, the hemolytic activity in this assay system of the proteins purified by methods described above measured $(1.9–2.5) \cdot 10^6$ CH50/mg (C8) and $(7.6–8.9) \cdot 10^5$ CH50/mg (C9).

Radioiodination of C8 and C9

Radiolabeling of C8 with Na^{131}I and C9 with Na^{125}I was performed in azide-free phosphate-buffered saline at 0°C using the solid phase glucose oxidase-lactoperoxidase system (enzymobeads, Bio-Rad). Unreacted radiolabel was removed by chromatography on Sephadex G-25 (PD-10 column, Pharmacia). Fatty acid-free bovine albumin (Sigma) carrier was added to 2% (w/v). Specific activities of $(0.1–7) \cdot 10^6$ cpm/μg protein were acheived. The functional activity of each radiolabeled protein was evaluated by the following criteria: (i) hemolytic assay (Cordis Laboratories, Miami, FL); (ii) specific incorporation of the labeled protein into membrane bound C5b-9, using C5b67 resealed erythrocyte ghosts as the target membrane (see below); and, (iii) sucrose density cushion floatation of C5b67 resealed erythrocyte ghosts exposed to limiting quantities of ^{131}I-C8 (or unlabeled C8) and excess C9, or, to limiting quantities of ^{125}I-C9 (or unlabeled C9) and excess C8 (see below). By these criteria, ^{131}I-C8 and ^{125}I-C9 used in these experiments retained greater than 90% of their hemolytic activity, and greater than 95% of their capacity to form sucrose-permeant lesions in erythrocyte ghost membranes as compared to equal molar quantities of the respective unlabeled protein. Binding activity of ^{131}I-C8 and ^{125}I-C9 to C5b67 ghosts was observed to range from 62 to 78% of input when each labeled protein was present at limiting concentrations. The specific binding of the unlabeled proteins was not determined. Specific binding of ^{125}I-C9 to C5b67 ghosts in the presence of ^{131}I-C8 was indistinguishable from that measured in the presence of equivalent molar quantities of unlabeled C8.

Resealed erythrocyte ghosts

Resealed ghost membranes of human erythrocytes (ghosts) were prepared by reversible hypotonic hemolysis at 0°C in the presence of 4 mM $MgSO_4$ according to published procedures [3–5,21].

Following restoration of isotonicity (165 mM KCl/2 mM Tris, pH 7.0–7.2) and resealing (60 min, 37°C), the sucrose-impermeant ghosts were isolated by density floatation (90 min, 45 000 × *g*) on a sucrose cushion. After washing in KCl-Tris, the ghosts were suspended to $5.0 \cdot 10^9$ ml^{-1} in the same solution. The ghosts were resealed to and retained 10–14 g hemoglobin per liter cell H_2O. For certain experiments, trace quantities (0.5–1 mCi per liter cell H_2O) of [^{14}C]sucrose was incorporated into the ghosts at the time of resealing. After resealing, the ghosts were maintained at 0–4°C and used within 18 h.

C5b67 ghosts 100 μg C7 was added to each ml of ghost suspension ($5 \cdot 10^9$ cells) and the ghosts then rapidly mixed with an equal volume of KCL-Tris containing 0–135 μg/ml C5b6. Following incubation (15 min, 37°C) the suspension was placed on ice, diluted with ice-cold KCl-Tris-albumin and centrifuged (5 min, 45 000 × *g*) at 2°C. After two additional washes with 10 vols. ice cold KCl-Tris-albumin, the C5b67 ghosts were suspended to $2.5 \cdot 10^9$/ml in KCl-Tris-albumin and used immediately. C5b6 controls were prepared by identical procedures, except that C7 was omitted.

C5b-9 ghosts incorporating ^{131}I-C8 and ^{125}I-C9. C5b-9 ghosts were prepared by incubating (30 min, 37°C) 200 μl volumes of C5b67 ghosts ($5 \cdot 10^8$ cells) with various quantities of ^{131}I-C8 (0–1340 ng) and ^{125}I-C9 (0–630 ng) in a total volume of 0.5 ml KCl-Tris-albumin. In certain experiments, unlabeled C8 and/or C9 was substituted for the labeled protein. To minimize possible C5b-8 inactivation before C9 binding, the C5b67 ghosts were routinely suspended with C9 before C8 was added [27]. In all experiments, matched-pair controls were prepared by suspending C5b6 controls with ^{131}I-C8 (or C8) and ^{125}I-C9 (or C9) under identical conditions. After incubation with C8 and C9, a 50 μl aliquot of each cell suspension was saved, and the remaining cells packed by centrifugation (Beckman Microfuge 12, 15 min., 4°C). The supernatant was aspirated and each cell pellet washed at 4°C with 4 × 1 ml vols. of KCl-Tris-albumin, and then suspended to 1 ml in the same buffer. 200 μl aliquots of each C5b-9 ghost suspension (and matched-pair control) were saved for measurement of cell count and cell-bound radioactivity and the remainder applied to discontinuous density gradients formed of 4 ml sucrose cushion overlayered with 2 ml KCl-Tris-albumin. Following centrifugation (90 min, 45 000 × *g*) ghosts floating at the buffer/sucrose cushion interface (sucrose-impermeant) were aspirated by pipet, and cell number and cell-bound radioactivity measured. The remaining buffer and sucrose cushion were then removed by aspiration, avoiding loss of the sedimented ghost pellet, and the sides of the tubes were wiped dry with a cotton swab. The sedimented ghosts (sucrose-permeant cells) were recovered in 1 ml and cell number and cell-bound radioactivity measured. By these techniques, total recovery of cells and cell-bound radioactivity (^{131}I-C8 and/or ^{125}I-C9) from the sucrose cushion (sum of sucrose-impermeant and sucrose-permeant fractions) generally exceeded 90% of the total applied ghost suspension.

Determination of C5b67-specific uptake of ^{131}I-C8 and ^{125}I-C9

The specific uptake of ^{131}I-C8 and ^{125}I-C9 by C5b67 ghosts was determined for each ghost suspension (before separation on sucrose cushions) – and for the density-cushion separated sucrose-impermeant and sucrose-permeant subpopulations of each suspension – on the basis of measured cell-bound radioactivity and cell number, after correction for nonspecific binding of ^{131}I-C8 and ^{125}I-C9 to corresponding, matched-pair C5b6 controls according to the following equation:

$$\text{Molecules bound/cell} = \frac{\text{dpm}^* \times \left(1 - \frac{\text{dpm}^0}{\text{dpm}^T}\right) \times N_A}{\gamma \times n_c}$$

where dpm* is measured radioactivity (^{131}I or ^{125}I) bound to C5b67 cells, dpm^0 is measured radioactivity bound to C5b6 controls, dpm^T is added radioactivity (^{131}I-C8 or ^{125}I-C9), and n_c^* is cell number determined for each sample. N_A is Avogadro's number and γ is the specific activity of each labeled protein expressed in dpm/mol.

Measured binding of ^{131}I-C8 to C5b6 controls, was always less than 0.5% of input. Measured binding of ^{125}I-C9 to C5b6 controls was observed to vary between 0.5–0.7% of input. Elevated nonspecific binding of ^{125}I-C9 (as compared to ^{131}I-C8) was similarly noted for control ghosts not exposed

to other complement proteins, and was not significantly reduced by additional washing.

Comparison of measured [^{14}C]sucrose release from C5b-9 ghosts to the distribution of the cells on sucrose density cushions

Ghosts resealed to [^{14}C]sucrose were made C5b67 and then suspended with various quantities of C8 and C9 (unlabeled) under conditions described above. Following incubation (60 min, 37°C), the tubes were centrifuged, supernatants removed, and the ghosts washed and applied to discontinuous sucrose density gradients under conditions identical to those previously described. Following centrifugation (90 min, 45 000 × *g*) the floating and sedimenting populations were recovered as previously described. Percent release of [^{14}C]sucrose for each C5b-9 cell suspension was determined from measured radioactivity in the cell-free supernatants, after correction for background release (2–6%) measured for matched-pair C5b6 controls. The amount of [^{14}C]sucrose retained by sucrose-cushion floating and sedimenting subpopulations of each C5b-9 ghost suspension was determined directly from the measured radioactivity in the cell water of ghosts recovered from the top and bottom of the density cushion, respectively. All samples were dissolved in 1% (v/v) Nonidet P-40 (Sigma) before scintillation spectrophotometry.

Assay. Hemoglobin concentrations were determined spectrophotometrically at 415 nm. Cell concentrations were determined by resistive counting in filtered saline (Coulter S-Plus) and from measured hemoglobin, utilizing a mean corpuscular volume of 94.1 fl and cell water of 969 g/kg ghosts [35]. Gamma counting was performed using a Beckman Gamma 4000 with corrections made for ^{131}I/^{125}I cross channel efficiencies. Liquid scintillation spectrophotometry was performed in 10 ml vols. Aquasol 2 (New England Nuclear) using a Beckman LSC8000.

Results

Separation of C5b-9 ghosts into sucrose-permeant and sucrose-impermeant populations

The goal of this study was to determine how many C5b-9 complexes must bind to the human erythrocyte ghost membrane to form a sucrose-permeant lesion and to determine whether the threshold in C5b-9 binding observed for onset of pore function is influenced by the average C9/C8 ratio of membrane-bound complexes. In these experiments, resealed erythrocyte ghosts maintained at a non-lytic osmotic steady-state served as the target cell. As previously described, the use of C5b-9 resealed ghost membranes permits direct analysis of the pore-forming properties of the C5b-9 proteins without subsequent colloid-osmotic expansion of cell water leading to lytic membrane rupture [36]. To relate onset of C5b-9 pore function to the quantitative membrane binding of these proteins per se, a discontinuous sucrose density barrier floatation method was employed to separate C5b-9 ghosts made permeant to sucrose from

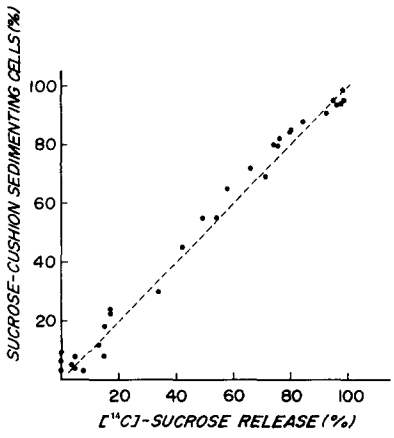

Fig. 1. Separation of sucrose-permeant and sucrose-impermeant C5b-9 ghosts by sucrose density centrifugation. Ghosts resealed to [^{14}C]sucrose were incubated with various quantities (0-10^4 molecules per cell) of the C5b-9 proteins (unlabeled) under conditions described in Materials and Methods. Following incubation, the C5b-9 specific release of radioactivity was determined (abscissa) and the cells layered on discontinuous density cushions formed of 43% (w/v) sucrose overlayered with KCl-Tris-albumin. Following centrifugation (90 min, 45000 × *g*) cells from the top (floating) and bottom (sedimenting) of the sucrose layer were each recovered, cell number determined, and radioactivity measured. The number of cells recovered from the bottom of the sucrose layer in each experiment (expressed as a percent of the total recovered cells) is plotted on the ordinate. In each experiment, total cell recovery (combined floating plus sedimenting) exceeded 90% of applied cell number. Data plotted on abscissa are corrected for nonspecific release of [^{14}C]sucrose measured for matched-pair C5b6 controls.

those ghosts that remained impermeant to this solute. This technique is a modification of the continuous density gradient methods previously employed by Bauer et al. [37], and permits quantitative recovery of both sucrose-permeant and sucrose-impermeant ghosts for determination of C5b-9 uptake by each group.

As shown by the data of Fig. 1, the number of ghosts sedimenting through the sucrose density barrier was found to correlate highly ($r = 0.99$) with the measured C5b-9-mediated release of [^{14}C]sucrose from the ghost water. Those C5b-9 treated ghosts which remained floating on the top of the sucrose layer retained 93 ± 17 (mean ± 2 S.D. percent) of [^{14}C]sucrose retained in the cell water of control ghosts, while ghosts sedimenting through the cushion retained 3 ± 2 (mean ± 2 S.D.) percent of this intracellular marker. These data confirm that cells recovered from the top and bottom of the sucrose density cushion represent sucrose-impermeant and sucrose-permeant subgroups of the total target population, respectively. Longer incubation of the C5b-9 treated ghosts, or, density centrifugation for longer than 90 min did not significantly alter either the measured total release of [^{14}C]sucrose, or, the distribution of the ghosts between the top and bottom of the sucrose layer. This suggests that the results obtained in these experiments reflect near-equilibria values. A detailed analysis of the kinetics of sucrose diffusion across the C5b-9 lesion in resealed ghost membranes has previously been presented [36].

Under the conditions of these experiments, lytic breakdown of the C5b-9-treated ghost membrane (as measured by hemoglobin release) did not significantly differ from that observed for controls. A small but significant increase in cell lysis above control levels was observed, however, when the input of the C5b-9 proteins exceeded amounts described in Materials and Methods (conditions resulting in the assembly of more than 10^4 C5b-9 sites/cell).

C8 and C9 binding to membrane C5b67 required for sucrose-permeant lesions

The number of C5b-9 complexes that must bind to the human erythrocyte membrane to inititate a change in its solute barrier properties was determined by directly relating the quantitative uptake of both ^{131}I-C8 and ^{125}I-C9 by C5b67-treated resealed ghosts to the number of these cells made permeant to sucrose (Fig. 2). In these experiments, the C5b67-specific binding of ^{131}I-C8 and ^{125}I-C9 to the ghost membrane (corrected for non specific uptake by control ghosts exposed to C5b6 but not C7) was used to determine the total number of C5b-9 complexes bound to the ghost membrane as well as the average C9/C5b-8 ratio within those complexes. Total C5b-9 uptake was altered either by varying the number of C5b67 sites in the presence of molar excess of both ^{131}I-C8 and ^{125}I-C9 (circles, Fig. 2), varying the input of ^{131}I-C8 to a fixed number of C5b67 sites in the presence of excess ^{125}I-C9 (squares, Fig. 2), or, by varying the input of ^{125}I-C9 to a fixed number of C5b67 sites

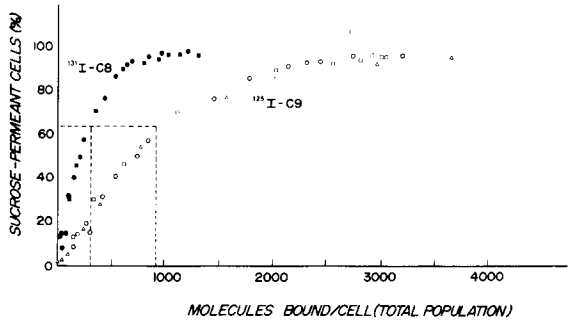

Fig. 2. Relationship of C5b67-specific uptake of ^{131}I-C8 and ^{125}I-C9 to the formation of sucrose-permeant membrane lesions. The number of C5b-9 ghosts made permeant to sucrose (ordinate) is plotted against the C5b67-specific uptake of ^{131}I-C8 and ^{125}I-C9 by the total target population (abscissa). Data plotted are combined results obtained for experiments performed under each of the following three conditions: i. Titration of C5b67: The number of effective C5b67 sites per ghost was varied between 0 and approx. 10^3/cell and each group of C5b67 cells exposed to 10^4 molecules/cell of both ^{131}I-C8 (●) and ^{125}I-C9 (○). ii. Titration of C8: ghosts were prepared with approx. 10^3 C5b67 sites/cell and exposed to various amounts (0–10^4 molecules/cell) ^{131}I-C8 (■) in addition to 10^4 molecules/cell ^{125}I-C9 (□). iii. Titration of C9: ghosts were prepared with approx. 10^3 C5b67 sites/cell and exposed to 10^4 molecules/cell ^{131}I-C8 and various amounts (0–10^4 molecules/cell) ^{125}I-C9 (△) Specific uptake of ^{131}I-C8 under these conditions measured 1034 ± 92 (mean ± 2 S.D.) molecules/cell (not plotted). In each experiment, the specific uptake of ^{131}I-C8 and ^{125}I-C9 by C5b67 ghosts was determined from cell-bound radioactivity measured after 30 min incubation (37°C), after correction for non specific uptake by matched-pair C5b6 controls. The number of sucrose-permeant cells was determined by centrifugation on density cushions formed of 43% (w/v) sucrose as described for Fig. 1.

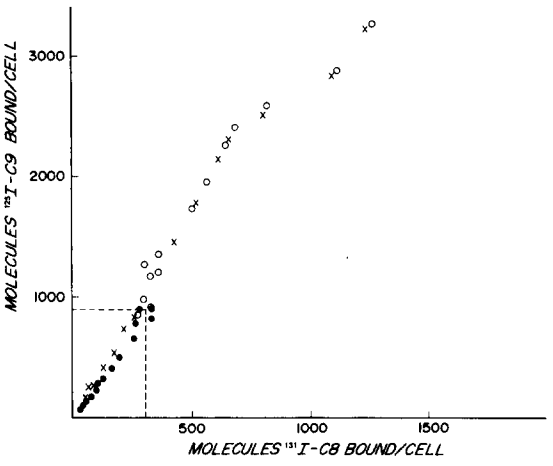

Fig. 3. Threshold of C5b-9 binding required for the formation of sucrose-permeant lesions: analysis by titration of C5b67 sites. The number of molecules of ^{131}I-C8 (abscissa) and ^{125}I-C9 (ordinate) specifically bound to C5b67 was determined for sucrose-permeant (○) and sucrose-impermeant (●) C5b-9 ghosts separated by centrifugation on sucrose density cushions. The specific uptake of each molecule by the total target population (before sucrose-cushion separation) is also plotted (X; from data of Fig. 2). Data plotted are results obtained for experiments in which ghosts expressing 0 to approx. 10^3 effective C5b67 sites/cell were exposed to 10^4 molecules/cell of both ^{131}I-C8 and ^{125}I-C9. Dashed lines are drawn at 300 (abscissa) and 900 (ordinate).

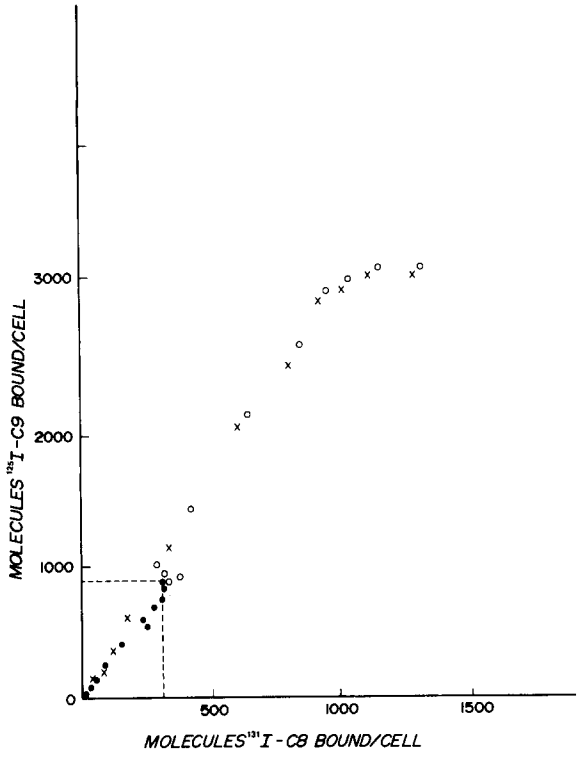

Fig. 4. Threshold of C5b-9 binding required for the formation of sucrose-permeant lesions: analysis by titration of C8. The number of molecules of ^{131}I-C8 (abscissa) and ^{125}I-C9 (ordinate) specifically bound to C5b67 was determined for sucrose-permeant (○) and sucrose-impermeant (●) C5b-9 ghost separated by centrifugation on sucrose density cushions. The specific uptake of each molecule by the total target population (before sucrose cushion separation) is also plotted (X, from data of Fig. 2). Data plotted are results obtained for experiments in which ghosts expressing approx. 10^3 effective C5b67 sites/cell were exposed to various amounts (0–10^4 molecules/cell) ^{131}I-C8, always in the presence of 10^4 molecules/cell ^{125}I-C9. Dashed lines are drawn at 300 (abscissa) and 900 (ordinate).

in the presence of excess ^{131}I-C8 (triangles, Fig. 2). In all experiments, washed C5b67 ghosts were suspended in the presence of ^{125}I-C9 before the addition of ^{131}I-C8. In each case, the C5b67-specific uptake of both ^{131}I-C8 and ^{125}I-C9 was directly measured, and at the same time, the number of these cells made permeant to sucrose was determined by density separation on 43% (w/v) sucrose (see Materials and Methods). As shown by the data plotted in Fig. 2, the formation of sucrose-permeant lesions in these membranes was observed to correlate to the C5b67-specific uptake of ^{131}I-C8 (in the presence of excess ^{125}I-C9) by a single hyperbolic function, and to the uptake of ^{125}I-C9 (under all experimental conditions) by a single, apparently non-hyperbolic function. If it is assumed that the distribution of the bound proteins among the target membranes conforms to a Poisson function (see Discussion) these data suggest that on the average approximately 300 C5b-8 complexes incorporating a total of 900 molecules of C9 must bind to these membranes before a sucrose permeant lesion is observed (dashed lines, Fig. 2). Over the range of molecular titrations employed in these experiments (see Materials and Methods) the ratio of membrane-bound ^{125}I-C9 to ^{131}I-C8 was observed to remain relatively invariant at approx. 3 : 1 (3.2 ± 0.8, mean ± 2 S.D.) under those experimental conditions for which the input of ^{125}I-C9 to bound C5b-8 was not made limiting to C9 uptake (circles and squares, Fig. 2).

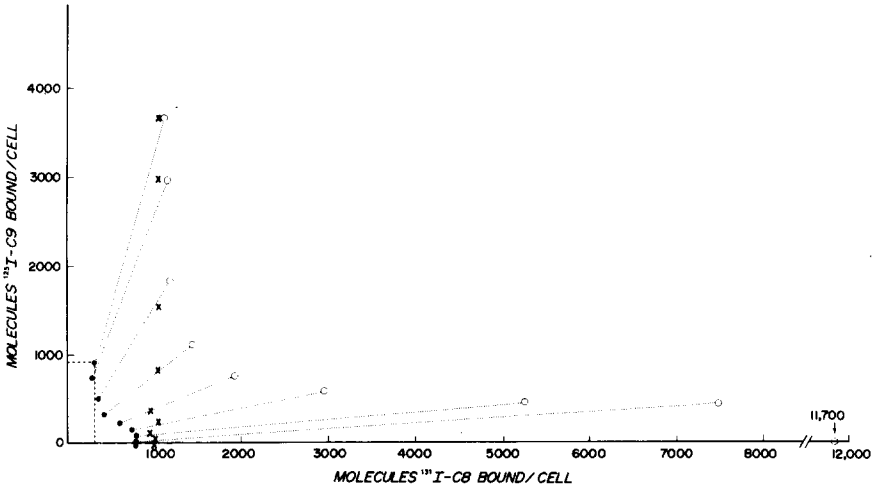

Fig. 5. Threshold of C5b-9 binding required for the formation of sucrose-permeant lesions: analysis by titration of C9. The number of molecules of ^{131}I-C8 (abscissa) and ^{125}I-C9 (ordinate) specifically bound to C5b67 was determined for sucrose-permeant (○) and sucrose-impermeant (●) C5b-9 ghosts separated by centrifugation on sucrose density cushions. The specific uptake of each molecule by the total target population (before sucrose cushion separation) is also plotted (X, from data of Fig. 2). Data plotted are results obtained for experiments in which ghosts expressing approx. 10^3 effective C5b67 sites/cell were exposed to various amounts ($0-10^4$ molecules/cell) ^{125}I-C9 always in the presence of 10^4 molecules/cell ^{131}I-C8. Dotted lines (●·····○) connect results obtained for sucrose-permeant and sucrose-impermeant subpopulations isolated from same initial C5b-9 ghost suspension. Dashed lines are drawn at 300 (abscissa) and 900 (ordinate).

Analysis of the molecular threshold for C5b-9 lesion formation

Following density centrifugation on sucrose cushions, the sucrose-impermeant (floating) and sucrose-permeant (sedimenting) subpopulations of the C5b-9 ghosts (prepared under the various conditions described for Fig. 2) were each recovered, and the C5b67-specific component of membrane-bound ^{131}I-C8 and ^{125}I-C9 determined for each group (see Materials and Methods). In Figs. 3 and 4 are plotted data obtained for the sucrose-impermeant and sucrose-permeant subpopulations of C5b-9 ghosts prepared under conditions of C5b-9 assembly for which the input of ^{125}I-C9 was always in molar excess to membrane-bound C5b-8 (total C5b-9 uptake varied by titration of either the number of cell-bound C5b67 sites or the input of ^{131}I-C8 in the presence of excess ^{125}I-C9). In Fig. 5 are plotted results obtained for sucrose-impermeant and sucrose-permeant subpopulations isolated from C5b-9 ghosts prepared under conditions for which the input of ^{125}I-C9 was progressively made limiting to C9 uptake. In each of these figures, the C5b67-specific component of cell-bound ^{131}I-C8 (abscissa) and ^{125}I-C9 (ordinate) are plotted for both the sucrose-permeant (open circles) and sucrose-impermeant (closed circles) subpopulations of the target cells. Also plotted is the measured uptake of each of these molecules by the total target C5b67 population, before separation of the cells by sucrose density centrifugation. Inspection of the data of Figs. 3–5 reveals that under each condition of C5b-9 assembly, the sucrose-permeant subpopulation of cells differ from those C5b-9 cells that remain impermeant to this solute by binding significantly greater amounts of both ^{131}I-C8 and ^{125}I-C9 per cell. For those C5b-9 cells prepared under conditions for which the input of ^{125}I-C9 was available in molar excess to the number of potential C5b-8 sites (see in particular data of Figs. 3 and 4), a threshold of approx. 300 C5b-8 complexes bound per cell (each incorporating approx. 3 molecules C9) appears to distinguish the sucrose-permeant from the sucrose-impermeant cells. Note that under the conditions of the experiments described for Figs. 3

and 4 (i.e., C5b-9 assembly in the presence of molar excess of ^{125}I-C9), the ratio of bound ^{125}I-C9 to bound ^{131}I-C8 for the sucrose-permeant subpopulation of ghosts (3.2 ± 0.7, mean ± 2 S.D.) does not significantly differ from that measured for the sucrose-impermeant ghosts (2.9 ± 0.6, mean ± 2 S.D.) and for both groups, remains generally invariant between 2 : 1 and 4 : 1 over the entire experimental range (cf. slopes of data plotted in Figs. 3 and 4).

In Fig. 5 are plotted results obtained for C5b-9 ghosts that were prepared under conditions for which the input of ^{125}I-C9 was progressively made limiting to C5b-9 assembly. Note that for each population of C5b-9 cells, the number of molecules of both ^{131}I-C8 and ^{125}I-C9 bound to the sucrose-permeant subgroup of cells (open circles) always exceeds that detected bound to the corresponding sucrose-inpermeant subgroup (closed circles). Under experimental conditions for which the molar ratio of ^{125}I-C9/^{131}I-C8 bound to the total cell population exceeds unity (i.e., ^{125}I-C9 uptake $> 10^3$ molecules/cell) the sucrose-permeant subgroup can be distinguished from the sucrose-impermeant C5b-9 subgroup on the basis of an apparent threshold of C5b-9 uptake representing approx. 300 molecules ^{131}I-C8 bound per cell. This corresponds to the threshold of total C5b-9 binding observed under the conditions described for Figs. 3 and 4. When the input of ^{125}I-C9 is made limiting to C5b-9 assembly (ratio of ^{125}I-C9/^{131}I-C8 bound per cell less than unity) the number of molecules of ^{131}I-C8 detected on the sucrose-permeant subgroup is observed to increase as a steep monotic funtion (Fig. 5). Under these conditions (bound ^{125}I-C9/^{131}I-C8 less than unity), the number of molecules of ^{131}I-C8 on the sucrose-impermeant cells increases above 300 per cell, suggesting an increase in the threshold of total C5b-8 uptake required to form a sucrose-permeant lesion. In the complete absence of C9 (abscissa, Fig. 5), the C5b67-specific uptake of ^{131}I-C8 by those cells made permeant to sucrose (representing 2% of the total population, see Fig. 2) was 11 700 molecules per cell. By comparison, an average of 764 molecules ^{131}I-C8 were measured bound per cell to the sucrose-impermeant subset under these conditions.

Discussion

The results of the present study suggest that a distinct threshold in total C5b-9 binding is required for the formation of a sucrose-permeant lesion in the human erythrocyte membrane: membranes to which fewer than 300 C5b-9 complexes are bound remain impermeant to sucrose, while those bearing more than 300 C5b-9 complexes are invariably permeable to this solute. When measured under a variety of conditions of C5b-9 assembly, the C9/C8 ratio of C5b-9 bound to cells made permeable to sucrose does not significantly differ from that observed for the subpopulation of cells that remain sucrose-impermeant. Taken together, these results suggest that expression of the functional activity of these proteins is related to the total density of assembled C5b-9 complexes bound to the membrane, and not directly to the multiplicity ($n > 1$) of C9 incorporated into each C5b-9 complex.

In previous reports (see for example, Refs. 8, 12, 19–25), 'dose-response' titrations of the C-proteins have generally been performed by relating measured membrane damage (detected either by red cell hemolysis or by the release of trace-labeled cell solute from resealed ghosts) to the relative input of each of the C-proteins. On the basis of the hyperbolic dose-response relationship observed for titrations of components C5b6-C8 (in the presence of excess C9), it has been the general conclusion of these studies that membrane damage occurs by a one-hit mechanism [8, 12, 19, 23]. This has been interpreted in molecular terms to mean that a single C5b-9 complex is sufficient to form a functional pore through the membrane [8, 19, 23]. These analyses were each based on the assumption that C5b-9 binding to the target cells is binomially distributed, and can be related to measured changes in membrane function (e.g., hemolysis or solute leak) by a Poisson relationship (i.e., it was assumed that the measured event occurred as the result of discrete, independent, and indistinguishable molecular events randomly distributed among the target cells). In the present study, the pore-forming activity of these proteins (experimentally derived on the basis of sucrose permeation) has been related directly to the quantitative membrane binding of the C5b-9 proteins per se (see Fig. 2). It

is interesting to note that under the conditions of these experiments, membrane pore formation is observed to correlate to C5b-9 binding (measured by C5b67-specific ^{131}I-C8 uptake in the presence of excess C9) by a single apparently hyperbolic function, suggesting prima facie a one-hit process. Nevertheless, these data also reveal that the unit event associated with the onset of sucrose permeation corresponds in molecular terms to the binding of approx. 300 C5b-9 complexes to each membrane. One plausible explanation for this result is that only 0.3% of every 300 C5b-9 complexes that bind to each membrane are functionally active and therefore, on the average, only one in three hundred forms an effective pore site in the membrane. Similar proposals have previously been made to account for the large number of 'doughnut-ring' lesions detected by electron microscopy on membranes lysed under conditions for which an average of only a single lesion per cell is predicted from analysis of complement lysis as a one-hit process (reviewed in Ref. 38). Alternatively, one can suggest that the measured unit event – in this case, conversion from the 'sucrose-impermeant' to 'sucrose-permeant' state – occurs when the density of C5b-9 complexes bound to any single membrane reaches a critical threshold level (i.e., > 300 per cell). This latter interpretation is particularly suggested by evidence that those membranes which remain impermeant to sucrose after C5b-9 assembly do exhibit increased permeability to molecules smaller than sucrose [9, 21]. Graded changes in effective C5b-9 pore size observed with increased C5b-9 binding is not expected if it is assumed that each membrane pore is initiated by a 'one-hit' process, constituting a single, discrete and independent even that leads to an all-or-nothing alteration of membrane function. Nevertheless, it is also possible that graded increases in membrane pore size observed with increased C5b-9 input may be due to structural variations within single, discrete C5b-9 complexes, that occur with probabilities inversely related to effective pore size.

In a previous study, Bauer et al. [37] demonstrated that after lysis of sheep red blood cells by antibody and human serum, the number of C5b-9 complexes bound to the lysed membranes (measured by ^{125}I-C7 uptake) correlates with the capacity of the membranes to subsequently reseal and regain their biconcave shape. In those experiments, C-lysed sheep red blood cells membranes bearing less than 850 C5b-9 complexes were observed to reseal to sucrose and regain a biconcave shape, while those C-lysed membranes bearing more than 850 complexes were invariably spheroid and remained leaky to sucrose. On the basis of these observations, they conclude that above a critical density of C5b-9 binding, there is a fundamental alteration of the erythrocyte membrane, leading to spheroidization and loss of the membrane's capacity to reseal. The results of the present study demonstrate that a critical threshold of C5b-9 binding is also required to initiate solute flow across the erythrocyte membrane, suggesting that the lytic activity of these proteins may also derive from the cumulative effects of multiple C5b-9 complexes bound to the same membrane. The interesting suggestion made by these authors that membrane damage by multiple C5b-9 results from a direct interaction of these molecules with intrinsic membrane proteins – leading eventually to cytoskeletal disruption – requires further study [37].

In a recent paper, Podack et al. [27] investigated morphological features of the C5b-9 proteins when bound to rabbit erythrocyte membranes at various molar ratios. In their experiments, molar ratios of C9/C8 as high as 16 : 1 were reported for membrane-bound C5b-9. Polymerization of C9 into distinct ring-like structures was observed at molar ratios of membrane-bound C9/C8 exceeding 6 : 1. At C9/C8 ratios of 3 : 1 or less, C5b-9 complexes on the membrane appeared to form condensed, aggregated structures, suggesting C5b-8 crosslinking by C9. The suggestion that dimeric crosslinks between molecules of C9 may mediate the aggregation of multiple C5b-9 complexes has also been made by Ware and Kolb [28]. It is of interest to note that in the present experiments (using human erythrocyte membranes), the ratio of C9/C8 within membrane C5b-9 was never observed to exceed 3–4 : 1, even when the input of C9 exceeded the number of potential C5b-8 sites by more than 100 : 1 (Figs. 2–4). A similar ratio of membrane-bound C9/C8 has been observed by Kolb and Muller-Eberhard [39]. One might speculate that the threshold of total C5b-9 binding (300 per cell) required to observe the pore-forming properties of

these proteins when bound to human erythrocyte membranes may relate to a restricted multiplicity of C9 binding to C5b-8 sites assembled on these membranes. The spontaneous loss of potential C9 hemolytic sites associated with the assembly of human C5b-8 on human erythrocytes can be inferred from the data of a recent study by Packman et al. [40].

As illustrated by the data of Fig. 5, the apparent threshold of C5b-8 binding required to form a sucrose-permeant lesion was observed to rise sharply when the C9/C8 ratio of the membrane-bound proteins was reduced below unity (by restricting C9 input). One possible explanation for this observation is that C9 acts directly to promote specific cooperative interactions among multiple C5b-8 complexes, for example, by aggregating the complexes into local clusters in the membrane, thereby increasing the local concentration of the membrane-bound proteins. Direct evidence for a C5b-8 cross-linking function of C9 has previously been presented [27, 28]. In the absence of such a specific cross-linking agent, the total surface density of C5b-8 required to achieve comparable aggregation states (e.g., through random collisional events) would be expected to increase dramatically. In this context, it is of interest to note recent data which suggest that the capacity of C5b-8 to increase membrane permeability to sucrose may be kinetically restricted by the rate of pore formation after C5b-8 binding [41]. Alternatively, the exposure of hydrophobic domains within C9 upon its association with C5b-8 is likely to alter the physical properties of the assembled complex, reducing the apparent threshold of total C5b-9 binding required to observe membrane damage. Evidence for the membrane insertion of hydrophobic domains of C9 [5, 33] and for a direct membranolytic activity associated with polymeric forms of C9 generated upon binding to C5b-8 has been presented [27, 34, 42]. Additional insight into how C9 enhances the lytic efficiency of the assembled C5b-9 proteins awaits further clarification of the relative contributions made by the 'C5b-8 cross-linking' and intrinsic membranolytic activities of this molecule.

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