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Transmembrane channel formation by complement: Functional analysis of the number of C5b6, C7, C8, and C9 molecules required for a single channel

(complement channels/channel size/channel structure/channel heterogeneity)

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ABSTRACT **Earlier studies have shown that sequential treatment of resealed erythrocyte ghosts with C5b6, C7, C8, and C9 leads to insertion of hydrophobic peptides from these complement proteins into the membrane and assembly of transmembrane channels. The number of molecules of each of the proteins required for assembly of the membrane-associated channel structure was evaluated by measuring the quantitative relationship between the doses of the individual proteins and the release of two trapped markers, sucrose and inulin, from ghosts after channel formation. The incubation period was sufficient to attain equilibrium of marker distribution between the ghosts and the extracellular fluid. Two markers of different size (sucrose and inulin, 0.9 and 3 nm molecular diameter, respectively) were used in order to develop information on the molecular composition of small and large channels, respectively. We found that participation of C5b6, C7, and C8 in channel formation displayed one-hit characteristics, regardless of marker size. By contrast, the participation of C9 was one-hit with respect to the sucrose marker, whereas with respect to the inulin marker the C9 reaction was multi-hit. Our results are compatible with the view that these markers are released through a channel structure in the membrane that is a monomer of C5b–9 of the composition $C5b6_1C7_1C8_1C9_n$, in which $n = 1$ for channels permitting passage of sucrose and $n = 2$ for channels allowing transit of inulin.**

Membrane attack by complement is initiated when complement protein C5 is cleaved into C5a and C5b by the C5 convertase of either the classical or the alternative activation pathway (1). The subsequent interactions among the terminal complement proteins (C5b–C9) are accompanied by exposure of hydrophobic peptides (2–4). If this occurs in the immediate vicinity of a bilayer membrane, some of the exposed peptides become inserted in the lipid bilayer (5–8) and are assembled into transmembrane channels (9–12).

From measurements of the molecular ratios among the individual complement proteins in the membrane-associated complex, Kolb *et al.* (13) proposed the formula $C5b_1C6_1C7_1C8_1C9_n$, with a maximal value of $n = 6$. Subsequently, Biesecker *et al.* (14) estimated from hydrodynamic analyses of detergent-extracted complexes that the molecular weight is 1.7×10^6. They concluded from this value that the complex is a dimer of C5b–9 and proposed the formula $(C5b_1C6_1C7_1C8_1C9_3)_2$.

By contrast, Bhakdi and Tranum-Jensen proposed that the complex is a monomer of C5b–9. They drew attention to the polydispersity of the detergent-solubilized complexes as judged by electron microscopy, ultracentrifugation in density gradients, and gel exclusion chromatography (15). According to their results, the smallest complexes are monomers of C5b–9, which have the structural appearance of hollow cylinders apparently identical to the characteristic complement lesions seen on target membranes. Their heavier ultracentrifugal fractions contained aggregates of these cylinders.

An important part of this disagreement derives from differences in the evaluation and interpretation of the hydrodynamic data on which the molecular weight estimates are based (14–16). In addition, it cannot be assumed *a priori* that the composition of C5b–9 complexes in detergent extracts is necessarily the same as that of membrane-associated complexes, especially because Ware *et al.* (16) have observed that the relative proportions among monomers, dimers, and trimers (or possibly tetramers) in the detergent extracts varied depending on the detergent used for solubilization and the experimental conditions.

Instead of pursuing this issue further by chemical studies, we now present direct functional assays of the number of molecules of each of the complement proteins required for assembly of the membrane-associated channel structure. This was done by measuring the quantitative relationship between the doses of the individual proteins and the amount of trapped marker released from resealed erythrocyte ghosts after channel formation. (Hereafter, "ghosts" refers to such resealed ghosts.) In order to exclude the influence of kinetic factors, the ghosts carrying complement channels were incubated for a period of time sufficient to attain equilibrium of marker distribution between the ghosts and the extracellular fluid. Because it is known that complement channels are heterogeneous with respect to size (12, 17, 18), two markers of different size (sucrose and inulin, 0.9 and 3 nm molecular diameter,* respectively) were used in order to obtain information on the molecular composition of channel structures that allow transit of a small or a larger marker, respectively.

A second issue addressed in the present work concerns the cause of the size heterogeneity of the complement channels. Boyle *et al.* (12) presented observations indicating that the size increases with the number of C9 molecules per C5b–9 complex. By contrast, Sims and Lauf (18), as well as Sims (20), claimed that channel size depends on the number of C5b–9 complexes per cell; this proposal involves the concept that large channels are formed from small ones by combination of two or more C5b–9 units to form multi-(C5b–9) complexes. A math-

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Abbreviations: C, complement; GVB^{++}, gelatin/Veronal buffered saline with Ca^{2+} and Mg^{2+}; $DGVB^{++}$, dextrose/gelatin/Veronal buffered saline with Ca^{2+} and Mg^{2+}; Hepes buffer, Hepes/glucose/NaCl/KCl/K_2HPO_4/Na_2HPO_4; $Hepes^{++}$ buffer, Hepes buffer with Ca^{2+} and Mg^{2+}; A, anti-Forssman antigen IgM; EA, sheep erythrocytes sensitized with anti-Forssman antigen IgM; K76 COONa, sodium salt of K76 monocarboxylic acid.

* Stokes radii for sucrose (0.44 nm) and inulin (1.52 nm) were obtained from Pappenheimer (19).

ematical analysis by DeLisi *et al.* (21) incorporated both factors—i.e., variation of C9 per C5b–9 complex and variation of C5b–9 per multi-(C5b–9)—to explain channel size heterogeneity.

MATERIALS AND METHODS

Buffers and Solutions. *Phosphate-buffered saline.* This buffer contained 150 mM NaCl and 5 mM sodium phosphate. The pH was adjusted to 7.4.

GVB^{++}. Veronal (barbital)-buffered saline, pH 7.4, containing 142 mM NaCl, 4.9 mM sodium Veronal, 0.1% gelatin, 0.15 mM $CaCl_2$, and 1 mM $MgCl_2$ was made by adding $CaCl_2$ and $MgCl_2$ to buffer A described in ref. 7.

$DGVB^{++}$. Veronal-buffered saline containing 71 mM NaCl and 2.5 mM sodium Veronal, and including gelatin, Ca^{2+}, and Mg^{2+}, was prepared with the incorporation of glucose (dextrose) to a final concentration of 139 mM as described for buffer B^{++} in ref. 7.

Mg^{2+}/acetate solution. This solution contained 4 mM $MgCl_2$ and 3.5 mM acetic acid. The pH was adjusted to 6.2.

Ca^{2+}/Mg^{2+} solution. A 10-fold concentrated stock solution was prepared by dissolving 1.9 g of $CaCl_2 \cdot 2H_2O$ and 2.0 g of $MgSO_4 \cdot 7H_2O$ in water and diluting up to 1.00 liter with water.

Hepes buffer. A 10-fold concentrated stock solution was prepared by dissolving 23.8 g of Hepes (Sigma), 18.0 g of glucose, 80.0 g of NaCl, 4.0 g of KCl, 0.68 g of K_2HPO_4, and 0.90 g of $Na_2HPO_4 \cdot 7H_2O$ in 900 ml of water, adjusting to pH 7.4 with 1 M NaOH solution, and diluting to 1.00 liter with water.

$Hepes^{++}$ buffer. Exactly 1 vol of Ca^{2+}/Mg^{2+} stock solution was mixed with 1.0 vol of the 10× concentrated Hepes stock and the mixture was adjusted with water to 10.0 vol.

Radioisotopes and Chemicals. *Radioisotopes.* [^{14}C]Sucrose (602 mCi/mmol, 0.1 mCi/ml; 1 Ci = 3.7×10^{10} becquerels) in ethanol/water, 9:1 (vol/vol), was purchased from New England Nuclear. Aliquots were dried and dissolved in Mg^{2+}/acetate solution immediately before use. [^{3}H]Inulin (0.34 mCi/mg) (average molecular weight 5,000) was purchased from New England Nuclear. Prior to use, the inulin was dissolved and chromatographed in phosphate-buffered saline, pH 7.4 on a polyacrylamide gel (Bio-Gel P-150, 100–200 mesh; Bio-Rad) column (1 × 70 cm) to eliminate any degraded low molecular weight material.

K76 monocarboxylic acid. The sodium salt of K76 monocarboxylic acid (K76 COONa) was kindly supplied by Otsuka Pharmaceutical (New York, NY).

Antibodies and Complement. *Antibodies.* Antibody to Forssman antigen was raised in rabbits and the IgM fraction was isolated as described in ref. 22.

Serum complement. Pooled fresh guinea pig serum as a source of complement was purchased from Rockland (Gilbertsville, PA). Human serum was obtained from outdated blood.

Purified C components. Human C5b6 was purified according to the procedure described in ref. 23. Guinea pig C7, C8, and C9 were prepared according to procedures described in ref. 22. There was no significant contamination of C8 in C9, and vice versa, as determined by hemolytic assays.

Procedures. *Preparation of EAC1,4b,2a,3b (EAC1–3b).* This intermediate was prepared by use of the C5 inhibitor K76 COONa according to the procedure of Hong *et al.* (24) with slight modification. One volume of a 1:1 mixture of guinea pig and human serum was agitated overnight at 0°C with 3 vol of K76 COONa at 4 mg/ml dissolved in $DGVB^{++}/GVB^{++}$ (1:1).

Antibody-sensitized sheep erythrocytes (EA) were suspended in $DGVB^{++}/GVB^{++}$ (1:1) at 3×10^9 cells per ml. One volume of EA was added to 2 vol of K76 COONa-treated serum and incubated for 60 min at 30°C with constant agitation. The cells were washed three times in $DGVB^{++}$ and resuspended in $DGVB^{++}/GVB^{++}$ (1:1) to 3×10^9 cells per ml. The incubation with K76 COONa-treated serum was repeated for 20 min at 30°C. The cells were washed four times in $DGVB^{++}$ and two times in phosphate-buffered saline, pH 7.4. The resulting EAC1–3b cells were immediately converted into ghosts by the procedure outlined below. (Alternatively, the EAC1–3b were converted to EAC1–7, as described below.) The titer of C5b6 on EAC1–3b prepared in this way was approximately 350 times greater than that with erythrocytes.

Conversion of EAC1–3b to EAC1–7. After two washings with GVB^{++}, the EAC1–3b were suspended in GVB^{++} to a concentration of 6×10^8 cells per ml. One hundred volumes of this suspension at 27°C was mixed with 1 vol of C5b6 (45,000 units/ml) and incubated for 30 min at 27°C. The cells were centrifuged and the pellet was resuspended in 100 vol of GVB^{++} at 27°C to which 1 vol of C7 (750,000 units/ml) had been added just prior to use. The suspension was then incubated for 50 min at 27°C. For storage, the cells were washed and suspended in $DGVB^{++}$. Alternatively, they were converted to ghosts as described below.

Preparation of ghosts and incorporation of markers. Ghosts were prepared from sheep erythrocytes in the state EAC1–3b or EAC1–7 by a modification of the method of Schwoch and Passow (25), as described in ref. 11. The erythrocytes were washed in phosphate-buffered saline, pH 7.4, and suspended to a concentration of 50% (vol/vol) in Hepes buffer. Two volumes of the 50% erythrocyte suspension at 0°C (±0.5°C) were mixed with 17 vol of ice-cold Mg^{2+}/acetate solution. After 5 min at 0°C, 1 vol of [^{14}C]sucrose and [^{3}H]inulin in Mg^{2+}/acetate solution was added and the pH was maintained at 6.2 for an additional 5 min at 0°C. Isotonicity was restored by addition of 2.4 vol of the 10× stock Hepes buffer and 2.4 vol of the Ca^{2+}/Mg^{2+} 10× stock. After another 5 min at 0°C, the mixture was incubated at 37°C for 50 min to reseal the ghosts. The ghosts were washed to remove untrapped markers, counted on a model B Coulter Counter, and adjusted to 3×10^9 ghosts per ml.

Dose–response measurements. Ghosts in $Hepes^{++}$, carrying C1–3b, were used for the C5b6 and C7 dose–response assays. Ghosts carrying C1–7 were used for the C8 and C9 assays. All analyses were made in triplicate with 250 μl of ghosts (3×10^9 per ml) in a total volume of 750 μl. The complete reaction mixtures were incubated at 37°C for 3.5 hr, the time required to reach the endpoint plateau of marker release. (Preliminary analyses with guinea pig serum C showed that kinetic marker release curves approached an endpoint plateau around 2.5 hr in experiments with 1×10^9 ghosts per ml. Additional experiments with purified terminal components, as in Figs. 1–4, showed that there was no significant difference in marker release between 3.0 and 4.5 hr with either marker, regardless of which of the terminal components was used at a limiting concentration.) After centrifugation, aliquots of the supernates were mixed with scintillation fluid [4 g of Omniscent (ICN) in 1 liter of 2:1 (vol/vol) toluene/Triton X-100] and the β emissions from ^{14}C and ^{3}H were counted in a Beckman LS-233 scintillation counter. (The radioactivities of the pellets were also determined as a check on marker recovery.) Each set of analyses consisted of triplicate background controls that contained ghosts, carrying C1–3b or C1–7, and all of the relevant complement proteins, except the one being titrated. The average marker release value of these background controls was subtracted from all of the measurements. The total amount of available marker was determined by lysing triplicate portions of ghosts with 2 vol of 1% Triton X-100, centrifuging, and measuring the released marker. Specific release (y) was calculated

as follows:

$$y = \frac{\text{experimental release} - \text{average background release}}{\text{average Triton X-100 release} - \text{average background release}}$$

The detailed conditions for the dose–response assays of C5b6, C7, C8, and C9 are given in the respective legends for Figs. 1–4. In each case, the complement components, other than the one being titrated, were used in "excess," which means that their concentrations could be shifted at least 2-fold in either direction without changing the results of the titration.

RESULTS

Dose–Response Data for C5b6. As described in the legend to Fig. 1, resealed sheep erythrocyte ghosts carrying C1–3b and containing trapped [^{14}C]sucrose and [^{3}H]inulin were treated with various dilutions of human C5b6 and excess guinea pig C7, followed by incubation with C8 and C9 for 3.5 hr at 37°C, the time required to reach the endpoint of marker release. The resulting dose–response curves are shown in Fig. 1 as plots of y (proportion of marker release) vs. the concentration of C5b6. The proportion of marker release represents the proportion of ghosts that carry at least one channel. It is evident that both of the curves are monotonic and pass through the origin. This indicates that only one molecule of C5b6 is required for formation of individual channels, regardless of marker size. It should be noted that the sucrose release curve lies substantially above the inulin curve, which indicates that some of the channels through which sucrose was released were too small for passage of inulin.

Dose–Response Data for C7. As described in the legend to Fig. 2, ghosts carrying C1–3b and containing trapped [^{14}C]sucrose and [^{3}H]inulin were incubated with various dilutions of C7 plus excess C5b6, C8, and C9. The results, assembled in Fig. 2, indicate that both curves are monotonic and pass through the origin. As in Fig. 1, there is evidence of heterogeneity with respect to channel size.

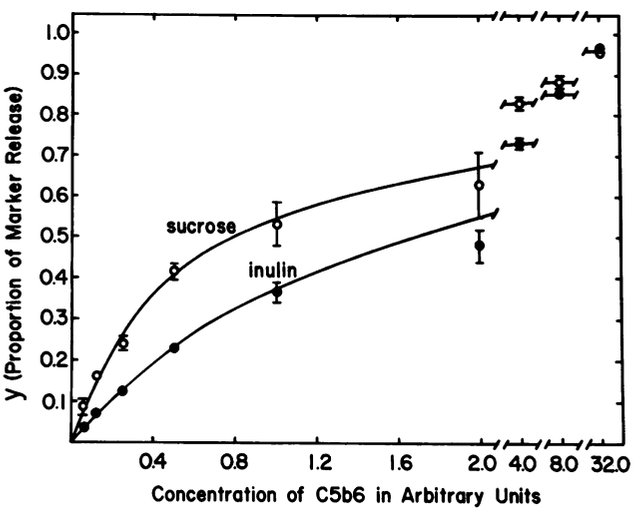

FIG. 1. Dose–response titrations of C5b6. Portions (125 μl) of a series of 2-fold dilutions of C5b6, ranging from 1:51,200 to 1:1,600, were incubated with 250 μl of ghosts carrying C1–3b (3×10^9 per ml) for 30 min at 27°C. Excess C7 (167 μl, 1.3×10^3 units/ml) was added and incubation was continued for 15 min at 27°C. After chilling to 0°C for a few minutes, 42 μl of C8 (1.2×10^4 units/ml), 42 μl of C9 (4×10^3 units/ml), and 124 μl of Hepes^{++} buffer were added, making a final reaction volume of 750 μl. The ordinate of the main part of the graph shows the mean of triplicate measurement (±1 SD) of the proportions of marker release, expressed as a fraction of 1. (The release of inulin and sucrose in the controls without C5b6 was 0.058 and 0.280, respectively.) The abscissa gives the concentration of C5b6 expressed in arbitrary units (1 = 1:3,200). In order to indicate that the curves approach complete marker release, points beyond the range of the curves are shown at C5b6 concentrations of 4.0, 8.0 and 32. Thus, the graph is discontinuous in this region, as indicated by the slashes.

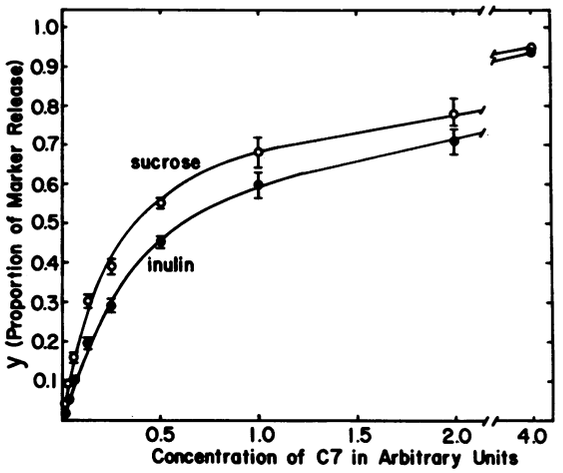

FIG. 2. Dose–response titrations of C7. Ghosts carrying C1–3b (250 μl) were incubated with 125 μl of C5b6 (8×10^3 units/ml) for 30 min at 27°C. Portions (167 μl) of a series of dilutions of C7 ranging from 1:800 to 1:204,800 (1 = 1:3,200 on the graph) were added and incubation was continued for 15 min at 27°C. After chilling at 0°C for a few minutes, 42 μl of C8 (1.2×10^4 units/ml), 42 μl of C9 (4×10^3 units/ml), and 124 μl of Hepes^{++} buffer were added. The results are plotted as indicated in Fig. 1. (The release of inulin and sucrose in the absence of C7 was 0.087 and 0.230, respectively.)

Dose–Response Data for C8. Ghosts carrying C1–7 and containing trapped [^{14}C]sucrose and [^{3}H]inulin were incubated with various dilutions of C8 and excess C9. Fig. 3 shows that both curves are monotonic and pass through the origin. As in Figs. 1 and 2 there is evidence of size heterogeneity.

Dose–Response Data for C9. Ghosts carrying C1–7 and containing trapped [^{14}C]sucrose and [^{3}H]inulin were treated with various dilutions of C9 and excess C8. The results in Fig. 4 show that the curve for sucrose is monotonic. By contrast, the curve

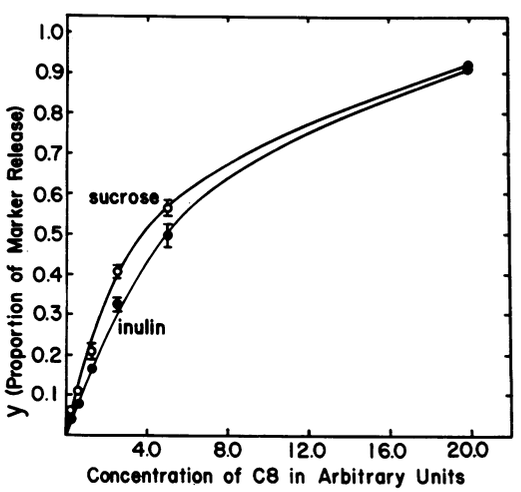

FIG. 3. Dose–response titrations of C8. A series of tubes was set up in an ice bath, each containing 250 μl of ghosts carrying C1–7 (3×10^9 per ml). Cold 42-μl portions of a series of dilutions of C8 ranging from 1:50 to 1:3,200 (1 = 1:1,000 on the graph) were introduced, and 42 μl of cold C9 (4×10^3 units/ml) as well as 416 μl of cold Hepes^{++} buffer were added. The results are plotted as indicated in Fig. 1. (The release of inulin and sucrose in the controls without C8 was 0.088 and 0.284, respectively.)

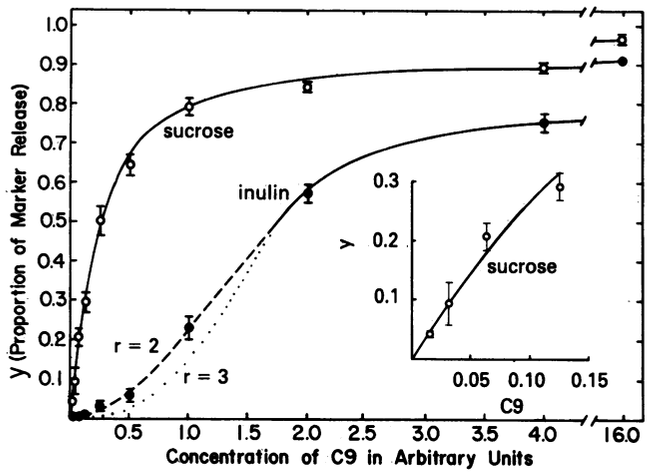

FIG. 4. Dose–response titrations of C9. A series of tubes was set up in an ice bath with 250 μl of ghosts carrying C1–7. Then 42 μl of cold C8 (1.2×10^4 units/ml) and cold 42-μl portions of a series of C9 dilutions ranging from 1:800 to 1:204,800 (1 = 1:3,200) were introduced, followed by 416 μl of cold $Hepes^{++}$ buffer. The results are plotted as for Fig. 1. (The release of inulin and sucrose in the controls without C9 was 0.054 and 0.360, respectively; in an additional control lacking C8 and C9, sucrose release was 0.234. The difference between 0.360 and 0.234 is attributed to the presence of C5b–8 channels that allow passage of sucrose, but not inulin.) For clearer display of the monotonic shape of the sucrose curve, an expanded plot is shown in the *Inset*. In order to determine how many molecules of C9 are required for an inulin channel, theoretical dose–response curves were constructed from the binomial distribution for critical thresholds (r) of 2 (broken line) or 3 (dotted line) molecules of C9, respectively. The theoretical curve for $r = 2$ was found to coincide with the experimental inulin release curve; both of these are shown as a broken line up to $y = 0.5$ marker release, the point at which the theoretical and experimental curves were made coincident by factorial adjustment of the abscissa. The theoretical curves were not extended beyond $y = 0.5$ because the upper part of the curve is not relevant to the critical threshold interpretation.

for inulin is sigmoidal, which indicates that two or more molecules of C9 are involved. Fig. 4 also shows two theoretical curves constructed from the binomial distribution for two and three molecules of C9. It is evident on inspection that the experimental data for the first half of the dose–response curve are concordant with a two-hit curve, but not with a three-hit curve.

DISCUSSION

As in other mechanistic studies, measurements of dose–response relationships have played a prominent part in complement research. The demonstration that the lysis of erythrocytes conforms to one-hit characteristics led directly to the proposal that complement forms transmembrane channels (26). Also, the one-hit theory put the functional assay of individual complement components on an absolute basis (27). Instead of the usual hemolytic assays, the present investigation of the dose–response relationships was done by measuring marker release from resealed erythrocyte ghosts because use of this method avoids difficulties inherent in the osmotic blocking procedure (21).

It is evident on inspection of Figs. 1–3 that all of the sucrose release curves are monotonic. Because the complement channels are heterogeneous with respect to size (12, 17, 18), each of these curves must be regarded as a composite representing a series of release curves covering the range of channel sizes that permit passage of sucrose. It is reasonable to assume that all, or at least most, of the constituent release curves that make up each composite curve are also monotonic. Therefore, we conclude from the shapes of the sucrose release curves in Figs. 1–3 that the formation of all or most of the channels allowing passage of sucrose is a one-hit process with respect to C5b6, C7, and C8. The same considerations are applicable to the inulin curves in Figs. 1–3, which indicate that the formation of all or most of the channels allowing passage of inulin is a one-hit process with respect to C5b6, C7, and C8. We interpret this to mean that a single molecule of each of these complement proteins is sufficient for assembly of the channel structures that permit passage of sucrose or inulin, even though these markers differ widely in size.

It is evident from Fig. 4 that the formation of sucrose-releasing channels is a one-hit process with respect to C9, whereas the assembly of inulin-releasing channels displays two-hit behavior. We have considered two alternative models that would explain the requirement for two molecules of C9. The first of these involves the concept that the second molecule of C9 is taken up by the $C5b_1C6_1C7_1C8_1C9_1$ complex, resulting in enlargement of the channel so that inulin can pass, in addition to sucrose. The alternative model entails the concept that two or more $C5b_1C6_1C7_1C8_1C9_1$ complexes combine to form a large multi-(C5b–9) channel. However, this hypothesis can be dismissed because Figs. 1–3 indicate that only one molecule each of C5b6, C7, and C8 is required for formation of inulin-releasing channels.

It is necessary to comment further on the segment of the sucrose curve in Fig. 4 between 0.5 and 2.0 arbitrary units of C9. This part is of interest because the number of inulin channels increases rapidly between these limits. Because sucrose can also pass through the inulin channels, it might be expected that the sharp rise in the inulin curve would cause a secondary rise in the sucrose curve. However, this is not the case because the uptake of a second molecule of C9 by the $C5b_1C6_1C7_1C8_1C9_1$ channel structure does not generate a new channel for transit of sucrose but merely enlarges the existing sucrose channel so that inulin can also pass. In fact, the sucrose release curve in Fig. 4 shows a pronounced flattening above 0.5 arbitrary unit of C9, which we would attribute to the diversion of some of the additional C9 molecules to channel enlargement rather than formation of new small channels.

Unfortunately, it is not possible to study the dose–response relationships with markers significantly larger than inulin for the reason that equilibration of such large markers between the ghosts and the extracellular fluid cannot be obtained within practicable periods of time. In light of this technical limitation, the conclusions that can be drawn from the present data with respect to the molecular composition of the complement channels are restricted to the size range of the two markers which we used. For this reason, we have no information relevant to either the 5.5- to 7.2-nm channels observed by Ramm and Mayer (17) or the even larger channels recently described by Dalmasso and Benson (28).

It is evident that our functional analysis based on sieving is in accord with the interpretation of Bhakdi and Tranum-Jensen's (15) chemical assays of detergent extracts. However, it is not compatible with the interpretation of Biesecker *et al.* (14), as well as that of Podack and Müller-Eberhard (29), who concluded from analyses of detergent extracts that the membrane-associated C5b–9 complex is a dimer. Moreover, as already indicated, our results speak against the multi-(C5b–9) model (18, 20) except, possibly, as a minor part of the total channel population. [The present observations are also incompatible with the recent claims by Podack and Tschopp (30) that poly(C9) forms the transmembrane channel.]

The Causes of Size Heterogeneity. Within the channel size limitations that have been stated, our results indicate that the

formula $C5b_1C6_1C7_1C8_1C9_n$ of Kolb *et al.* (13) represents the actual composition of the membrane-associated channels. When $n = 1$, the channels are small (*ca.* 1–3 nm), whereas $n = 2$ is applicable to bigger channels (>3 nm). (It remains to be determined whether the binding of a third, fourth, or fifth C9 molecule, etc. causes further channel enlargement.) Our results are in general accord with the proposal by Boyle *et al.* (12) that the size of the channels increases with input of C9, but we do not agree with the concept advanced by Sims and Lauf (18) and Sims (20) that channel size depends on the number of constituent C5b–9 units in multi-(C5b–9).

The mathematical model of size heterogeneity by DeLisi *et al.* (21) includes C9 variation in the C5b–9 complex, as well as C5b–9 variation in multi-(C5b–9) to explain channel size heterogeneity. Their evaluation involves the assumption that binding of one, two, or three C9 molecules will result in the formation of progressively larger channels that permit lysis of erythrocytes in a solution of NaCl (hydrated Na^+ Stokes radius is 0.14 nm), of glucose (Stokes radius 0.36 nm), or of raffinose (Stokes radius 0.56 nm), respectively. While we accept this model in general, we disagree with their estimates of channel sizes when one, two, or three C9 are bound to the C5b–9 complex; also, as indicated earlier, our data argue against inclusion of the multi-(C5b–9) concept.

While we support C9 multiplicity as a cause of size heterogeneity, our observations also indicate that this is not the only factor responsible for size variation. Thus, the dose–response curves in Figs. 1–3 show substantial differences between sucrose and inulin release even at very low levels of C5b6 (Fig. 1), C7 (Fig. 2), and C8 (Fig. 3), where the multiplicity of C9 relative to C8 would be large. This indicates that small channels do not disappear entirely when the input of C9 is large. Hence, complement channels do not approach a uniform large size at high C9 multiplicity, which means that factors other than C9 multiplicity also influence channel size. We do not know what these factors might be, but we suspect that random variations in channel geometry may be responsible.

Note Added in Proof. The multi-(C5b–9) model is described fully in an article by Esser (31).

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