The carbocyanine dye 3,3'-dipropylthiodicarbocyanine iodide has been used to investigate changes in membrane potential ($E_m$) which occur upon binding of complement proteins C5b-9 to the plasma membrane of blood platelets. Gel-filtered platelets exposed to C5b6 and C7 in serum-free medium show no change in $E_m$ from that of controls, as indicated by either 3,3'-dipropylthiodicarbocyanine iodide fluorescence or by the distribution of [14C]tetraphenylphosphonium bromide. Addition of complement proteins C8 and C9 to the C5b6C7 platelets results in partial depolarization of $E_m$, which spontaneously repolarizes to basal levels within 15-20 min at 37 °C. Under these conditions, C5b-9-treated platelets show no increase in lysis over complement-free controls. Isotonic replacement of external sodium by either potassium or choline alters both the rate and extent of membrane depolarization and inhibits the platelets' capacity to repolarize after C5b-9 assembly. Repolarization of $E_m$ to basal levels is also completely blocked by addition of ouabain, confirming that this recovery is mediated by the plasma membrane Na⁺/K⁺ pump. These results demonstrate that membrane binding of the C5b-9 proteins can induce a transient change in $E_m$ when bound to the plasma membrane at a sublytic concentration, providing a mechanism for target cell activation by these potentially cytolytic proteins.

Recent data pertaining to the physical properties of the complex composed of complement proteins C5b, C6, C7, C8, and C9 (reviewed in Refs. 1-3). The assembly of this complex from its five component glycoproteins initiates hydrophilic-to-hydrophobic conformational transitions within the proteins, which results in their insertion into membrane lipid with a concomitant increase in membrane permeability to aqueous solute. By undermining the solute barrier function of the plasma membrane, the C5b-9 proteins initiate the collapse of ionic gradients which ultimately leads to cell death (4, 5).

In vitro, C5b-9 binding to the platelet membrane can initiate a transient and reversible depolarization of the membrane potential ($E_m$), which occurs in the absence of cell lysis, and which is followed by a ouabain-inhibitable repolarization of $E_m$ to basal levels.

**EXPERIMENTAL PROCEDURES**

Materials—DiS-C3-5 was obtained from Molecular Probes (Junction City, OR) and as a most generous gift from Professor Alan S. Waggoner (Department of Biology, Carnegie-Mellon University); [14C]tetraphenylphosphonium bromide and Aquasol-2 were from New England Nuclear (Boston, MA). Valinomycin, ouabain, NADH, and sodium pyruvate were obtained from Sigma (St. Louis, MO). Dibutyl phthalate and bis(2-ethylhexyl)phthalate were from Eastman Chem...
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ics (Rochester, NY). All other chemicals were of reagent or analytical grade.

Solutions—All solutions were freshly prepared using deionized H2O obtained by reverse osmosis and ultrafiltration. Medium I contained 145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 0.5 mM sodium phosphate, 0.1 g/100 ml of dextrose, 5 mM PIPES, pH 6.8. Medium II contained 145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 0.5 mM sodium phosphate, 0.1 g/100 ml of dextrose, 5 mM HEPES, pH 7.4.

Platelets—Platelet concentrates were obtained from normal adult volunteers by plasmapheresis, using either an IBM Model 2997 or Ferrall Model CS3000, and used within 4 h of collection. Except where otherwise indicated, cells were maintained at room temperature, and plastic was used throughout. After an initial low-speed spin to remove contaminating red cells (4 min, 200 x g), the platelets were concentrated to 1-2 x 10^11/ml by gentle centrifugation (20 min, 500 x g), and 250 µl of this suspension was applied to a column (0.8 x 30 cm) of Sepharose CL-2B (Pharmacia) equilibrated in Medium I. The peak cell fraction eluting in the void (generally 1-3 x 10^10 cells/ml) was recovered for immediate use. Platelet suspensions obtained by these methods were completely devoid of contaminating serum C7, C8, or C9 activity, as measured by standard hemolytic assay using antibody-sensitized sheep red blood cells and human sera deficient in such classical pathway factors (see below).

Cell counting and adhesive sizing were performed using Coulter Model ZBI and Channelizer (C1000).

Complement Proteins—Human complement proteins C5b6, C7, C8, and C9 were purified and assayed for functional activity as previously described (19). Human sera deficient in one or more of these factors were dialyzed into Medium II and briefly warmed to room temperature. Human sera deficient in either C7, C8, or C9 were prepared by absorptions against the IgG fraction of goat monospecific antiserum raised against each protein, and these depleted sera were used for functional assay of the proteins, according to methods previously described (19, 21).

C5b67 Platelets—To assemble membrane-bound C5b67 complexes, gel-filtered platelets were suspended with C7 (5 µg/1 x 10^8 cells), and 15 µg of C5b6 was added with rapid mixing in a final volume of 150 µl. Following 15-min incubation at room temperature, the C5b67 platelets were recovered and used immediately. For all experiments, matched-pair controls were prepared by omitting C7 (C5b6 control) or by omitting both C5b6 and C7 (complement-free control).

Fluorescence Measurements—Changes in membrane potential accompanying C5b-9 assembly were monitored by use of the fluorescent dye indicator diS-C3-(5), according to a modification of published methods (22-24). In each experiment, 2.5 x 10^5 platelets (C5b67 or controls) were added to 2 ml of Medium II in a cuvette equilibrated at 37°C. For certain experiments, either C5 or choline Cl was substituted isotonically for NaCl in the suspending medium. Two microliters of diS-C3-(5) (5 µg/1 x 10^8 cells), and 15 µg of C5b6 was added with rapid mixing in a final volume of 150 µl. Following 15-min incubation at room temperature, the C5b67 platelets were recovered and used immediately. For all experiments, matched-pair controls were prepared by omitting C7 (C5b6 control) or by omitting both C5b6 and C7 (complement-free control).

RESULTS

We have previously demonstrated that the binding of complement proteins C5b-9 to the human erythrocyte initiates a depolarization of the membrane potential that occurs prior to the onset of hemolysis (21). In the case of the erythrocyte, this electrochemical collapse is irreversible and is unaffected by the activity of the sodium pump. Recent reports that assembly of these cytolysin serum proteins on blood platelets can lead to the thrombotic activation of the cells, rather than to plasma membrane rupture, led us to consider how the electrochemical changes induced by the C5b-9 proteins, when bound to platelets, might differ from those observed for complement-damaged erythrocytes. Of particular interest was the possibility that platelets (and presumably other cells) possess a mechanism for the restoration of a stable electrochemical steady state in the face of the increased ion conductance mediated by the membrane-inserted C5b-9 proteins, thereby enabling them to escape the cytolytic consequence of immune attack.

Change in E<sub>m</sub> Due to Membrane Binding of Complement Proteins C5b-9—As shown by the data of Fig. 1, platelets treated with C5b6 + C7 and maintained in plasma-free suspension at 37°C accumulate diS-C3-(5) to the same level of fluorescence as observed for controls. Based on fluorescence calibration performed by cell incubation at various external [KCl] and saturating concentrations of the potassium-selective ionophore valinomycin (see "Experimental Procedures"), the resting membrane potential of both control and C5b67 platelets (in the absence of added C8 or C9) was estimated to be -57 ± 6 mV, in good agreement with data previously reported for (untreated) platelets suspended under compara-
Membrane Potential of C5b-9-damaged Platelets

**TABLE I**

<table>
<thead>
<tr>
<th>C8 input</th>
<th>C9 input</th>
<th>C9/C8 molar ratio</th>
<th>$\Delta E_m$</th>
<th>LDH release</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>µg</td>
<td></td>
<td>µV</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.35</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0)%</td>
</tr>
<tr>
<td>C5b67</td>
<td>0.07</td>
<td>1.0</td>
<td>30.4</td>
<td>5.1%</td>
</tr>
<tr>
<td>0.14</td>
<td>1.0</td>
<td>0.14</td>
<td>15.2</td>
<td>7.3%</td>
</tr>
<tr>
<td>0.7</td>
<td>1.0</td>
<td>0.7</td>
<td>3.0</td>
<td>12.9%</td>
</tr>
<tr>
<td>1.4</td>
<td>1.0</td>
<td>1.4</td>
<td>1.5</td>
<td>15.5%</td>
</tr>
<tr>
<td>2.8</td>
<td>1.0</td>
<td>2.8</td>
<td>0.76</td>
<td>15.7%</td>
</tr>
</tbody>
</table>

* Molar ratios at input calculated on the basis of molecular weights of 151,000 (C8) and 71,000 (C9), respectively.
* Estimated from peak increase in fluorescence measured under conditions described for Fig. 1. Calibration of diS-Ca-(5) fluorescence to $E_m$ performed on the basis of Equation 1. See “Materials and Methods.”
* All values corrected for background release of lactic acid dehydrogenase (LDH) measured for complement-free controls.
* Parentheses indicate release less than or equal to that for complement-free controls.

![FIG. 1.](https://example.com/fig1.png)

Change in membrane potential due to C5b-9 assembly: Titration of C8 and C9. C5b67 platelets suspended in Medium II were equilibrated with diS-Ca-(5) at 37°C. At time indicated by arrow, C8 and C9 were added to the cuvette in amounts indicated below. Fluorescence measurements (left ordinate) and calibration of membrane potential (right ordinate) were performed according to methods described in text (see “Materials and Methods”). Panel A, all samples received 0.35 µg of C8 plus the following amounts of C9: 0 µg (-----), 0.1 µg (-----), 0.5 µg (-----), 1.0 µg (-----), 2.0 µg (-----), or 3.0 µg (-----). Results obtained for control platelets receiving 0.35 µg of C8 plus 3.0 µg of C9 are also shown (-----). Panel B, all samples received 1.0 µg of C9 plus the following amounts of C8: 0 µg (-----), 0.07 µg (-----), 0.14 µg (-----), 0.7 µg (-----), 1.4 µg (-----), or 2.8 µg (-----).

Differential ionic conditions (23, 24, 27). Because of a slow spontaneous depolarization of $E_m$ for C5b67 and control platelets during suspension in plasma-free media, all experiments were performed within 90 min of cell isolation by gel filtration.

Addition of both C8 and C9 to the C5b67 platelet suspension resulted in a dose-dependent increase in diS-Ca-(5) fluorescence, corresponding to maximal depolarization of the membrane potential by nearly +20 mV under the conditions of these experiments (Fig. 1 and Table I). The depolarization of membrane potential upon addition of C8 and C9 to C5b67 cells qualitatively conformed to results obtained previously with complement-treated erythrocytes, for which a collapse of $E_m$ (from –7 to 0 mV) upon C5b-9 assembly was detected prior to the onset of hemolysis (24). In the case of C5b-9 platelets approaching 0 mV at maximal input of the complement proteins (data not shown). Under these conditions (depolarization of $E_m$ by more than 20–30 mV), the membrane potential generally failed to repolarize to basal levels, suggesting that the cells’ capacity to reverse the electrochemical change mediated by the bound C5b-9 proteins is dependent upon the magnitude of the initial depolarizing conductance.

In the absence of added C9, a small increase in $E_m$ of the C5b67 platelets was observed upon C8 addition (see Fig. 1A and Table I), consistent with the known potential of C5b-8 complexes to alter membrane permeability when bound to the plasma membrane at high multiplicity (25). Again, C5b-8-mediated depolarization occurred without detectable cell lysis and was followed by a spontaneous recovery of the membrane potential to initial levels.

As shown by the data of Fig. 1A and Table I, diS-Ca-(5) fluorescence measured for control platelets was unaffected by the addition of both C8 and C9 to the cuvette, confirming that the response measured upon addition of these proteins to the C5b67 platelets arises specifically from a change in $E_m$ mediated by the membrane-bound C5b-9 complex per se and cannot be attributed either to a direct effect of C8 or C9 on the distribution and emission of the fluorophore, or to possible contamination of these proteins by another platelet mem-
brane-reactive substance (see “Discussion”).

Role of External Sodium—In order to gain insight into the individual ion conductances responsible for the change in $E_m$ following assembly of the C5b-9 proteins, experiments were performed under conditions of isotonic replacement of external sodium by either potassium (Fig. 2) or choline (Fig. 3). In the presence of increased external potassium, the basal $E_m$ of the platelet membrane (C5b67 or controls) was found to partially depolarize, presumably reflecting the contribution of the potassium equilibrium potential to the overall membrane potential of these cells (the permeability of the untreated platelet membrane to $K^+$ exceeding its permeability to $Na^+$; see Refs. 24 and 27). As is also shown by the data of Fig. 2, replacement of external sodium by potassium reduces the magnitude of the initial membrane depolarization observed upon C5b-9 assembly and also inhibits repolarization to the basal membrane potential ($E_m$ measured before C8/C9 additions). Comparison to the data obtained in the presence of external choline (Fig. 3) suggests that the altered response observed for suspension at high external potassium (i.e. a diminished C5b-9 specific membrane depolarization and an inhibition of the cell’s capacity to repolarize) is related to the decrease in external sodium under these conditions, rather than to an increase in external potassium per se. In light of our previous observation that membrane binding of the C5b-9 proteins is unaltered by these ion substitutions or by changes in membrane potential (20), these results suggest that the altered response of the cells when suspended in low-$Na^+$ media reflects differential ion conductances across the C5b-9-damaged plasma membrane, rather than an effect of these ions on the assembly of functional C5b-9 complexes per se. In this context, it is of interest to note that in addition to decreasing the magnitude of the response observed upon C8 and C9 additions to the C5b67 cells, substitution of external sodium by choline also decreases the initial rate of membrane depolarization, suggesting a preferential conductance of sodium versus choline across the C5b-9 pore (see “Discussion”). Evidence for ion-selective conductance mediated by the C5b-9 pore has also been acquired by single-channel measurements in lipid bilayer membranes.²

Recovery from C5b-9-induced Membrane Depolarization: Inhibition by Ouabain—The dependence of the observed response to C5b-9 assembly on external sodium suggested that the change in $E_m$ is related in large part to the increased conductance of this ion mediated by the C5b-9 pore (shifting $E_m$ towards the $Na^+$ equilibrium potential) which the cell compensates through a counteracting electrogenic conductance. Since the capacity of these cells to repolarize after C5b-9-induced depolarization of $E_m$ also required that sodium serve as the (inward) charge carrier, it seemed likely that the cells’ compensatory response is mediated by an electrogenic sodium-specific efflux. The participation of an active transport mechanism mediating the response to C5b-9-induced depolarization was also suggested by our observation that the ability of target platelets to reverse this electrochemical change is rapidly lost during in vitro storage of the cells, the biphasic response to C5b-9 assembly generally observed only for platelets used within 4–6 h after plasmapheresis and within 1–2 h after separation from plasma (data not shown). Accordingly, the contribution of stimulated active sodium efflux to the repolarization observed after C5b-9 binding was

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² R. Benz, T. Wiedmer, and P. J. Sims, manuscript in preparation.
directly investigated, using ouabain to inhibit the Na/K pump of the target C5b67 platelets. As shown by the data of Fig. 4, addition of ouabain to C5b67 platelets completely blocks their ability to recover in response to C8/C9-induced depolarization of \( E_m \), thereby confirming that active sodium transport mediates the membrane repolarization observed after C5b-9 binding. As previously reported for untreated platelets, the addition of ouabain to these cells (in the absence of C8 and C9) was also observed to depolarize the basal membrane potential by between 5 and 12 mV, corresponding to a reversal of that component of \( E_m \) normally contributed by the sodium pump, through its electrogenic Na/K exchange (27, 29).

**DISCUSSION**

The results of these experiments provide direct evidence that the C5b-9 proteins can sublytically modulate the electrochemical steady state of the plasma membrane, providing a mechanism by which these serum immunoproteins potentially induce activation of their target cell. Furthermore, these results provide insight into the mechanisms by which even anucleate hematologic cells can avoid the cytolytic consequence of immune attack, after C5 activation and the consequent assembly and insertion of functional C5b-9 pores into the plasma membrane.

It is of interest to consider how the electrochemical changes we measure for the C5b-9 damaged platelet membrane are related to previous observations regarding the potential for thrombotic activation of this cell consequent to its immune injury. As reported by Polley et al. (13, 15), C5b-9 can induce platelet aggregation in vitro and cause release of serotonin and thromboxane. Furthermore, there is circumstantial evidence for a role of these proteins in episodes of intravascular thrombosis that can occur in the setting of immunological disease. Based on the known mechanisms of signal transduction across the plasma membrane leading to cell activation, one can speculate that the C5b-9 proteins modulate platelet reactivity by one or more of a variety of mechanisms, including directly through the dissipation of transmembrane cation gradients. For example, Simons and colleagues have demonstrated that membrane depolarization (mediated by an amiloride-sensitive sodium influx) accompanies thrombin stimulation leading to the secretory response and aggregation (30). Although results from previous studies suggest that a depolarization of \( E_m \) is not sufficient to directly induce platelet secretion or aggregation (27), this change in membrane potential has been reported to potentiate the platelet's aggregation in response to adenosine diphosphate (29–31). On the other hand, elevation of cytoplasmic pH and ionized calcium—electrochemical changes which are likely to occur secondarily to plasma membrane damage—must also be considered in the overall cellular response to C5b-9 insertion. Evidence for a C5b-9-mediated calcium influx accompanying immune damage to the plasma membrane has recently been reported by Campbell and co-workers (16, 18). Finally, one might also speculate that a change in the lamellar configuration of membrane lipid, which is known to accompany the insertion of these large amphiphilic proteins (see Ref. 32), may itself promote the availability of substrate to intracellular phospholipases and/or phosphokinases, thereby elevating the concentration of endogenous intracellular mediators of calcium release and platelet activation.

Our data suggest that C5b-9 assembly on the platelet membrane results in a relatively small decrease in net membrane resistance (reflected by a partial depolarization of \( E_m \)), which the cell can compensate with a counterbalancing electrogenic conductance (mediated by the Na*/K* pump). Furthermore, the data of Figs. 2 and 3 suggest that there is a degree of selectivity to the ion conductance mediated by the C5b-9 pore (at least for Na* versus choline). These results are therefore consistent with data reported by Lauf (33) and Sims and Lauf (34) which suggest that the effective size of the membrane pores formed by the C5b-9 proteins, as measured either by the radius of solute exclusion or the kinetics of solute permeation, can be substantially smaller than the 10 nm, inner diameter, transmembrane tubule depicted by ultrastructural analysis of the assembled C5b-9 proteins. In the context of current speculation regarding the functional significance of this large membrane-embedded tubule, which has been shown by Müller-Eberhard and co-workers to be composed of a 12-18 subunit ring polymer of C9 that is generated during its interaction with membrane-bound C5b-8 (35–37), it is of interest to consider the electrochemical changes that would be expected to result from insertion of only a single such “channel” through the plasma membrane of a platelet. Based upon dimensions derived from electron micrographs of the poly-C9 tubule (approximately 10 nm inner diameter \( \times 20 \) nm length; Refs. 35–37) one would anticipate a net diffusional Na+ influx (and K+ efflux) across this transmembrane pore at an initial rate of \( 7 \times 10^{-16} \) mol/s. Considering a platelet volume of approximately 10 fl, this diffusional exchange would virtually dissipate the transmembrane gradient of these ions within the first 1–2 s. By contrast, the partial depolarization of \( E_m \) that we observe upon membrane assembly of these proteins requires several minutes to reach completion under conditions where the input of C8 and C9 to the C5b67 platelets is on the order of \( 10^6 \) to \( 10^7 \) cell, and even under conditions where the opposing active Na/K exchange is completely inhibited (cf. Figs. 1 and 4). Based upon considerations previously discussed (see Ref. 21), neither the kinetics of C8/C9 binding nor the rate of redistribution of the dye indicator are likely to be limiting to the rate of fluorescence change measured upon C8 and C9 additions to the C5b67 cells. On the other hand, the possibility remains that the change in \( E_m \) we observe after C8/C9 additions is kinetically limited by the activation process required for membrane pore formation after binding of the complement proteins, which presumably corresponds to the molecular events of C9 unfolding, self-polymerization, and membrane insertion. Although the kinetics of this C9 activation step have not been analyzed for C5b-9-treated platelet membranes, this process has been investigated for the C5b-9 proteins bound to lipid vesicles and sheep erythrocyte membranes (38). For example, on the basis of data obtained by fluorescence resonance energy transfer methods, we have previously demonstrated that C9 polymerization upon mixing with membrane-bound C5b-8 is virtually complete within the first 30 s at \( 37^\circ C \) (see Fig. 2 in Ref. 38).

Since under these conditions \( E_m \) of the target platelets requires several minutes to maximally depolarize, it does not appear that the time course of this measured electrochemical change reflects the kinetics of C5b-9 pore formation per se. Nevertheless, a direct measurement of the rate of this activation event for the C5b-9 proteins bound to human platelet membranes will be required to exclude this possibility.

Inspection of the data of Fig. 1 and Table I reveals that the depolarization of \( E_m \) observed upon addition of C8 and C9 is not directly related to the molar ratio of C9 to C8 per se, but rather to the net input of both of these proteins to the C5b67
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cells. These results therefore suggest that membrane conductance is increased by the cumulative effect of multiple membrane-bound C5b-9 complexes rather than by polymeric addition of C9 to a single discrete C5b-9 pore site. Similar conclusions have been reached by one of us previously, based upon analysis of how the solute exclusion radius of the membrane pore is related to the input of the individual C5b-9 proteins, as well as by direct measurement of the molecular stoichiometry of the membrane-bound complexes (7, 8, 10). Finally, it is of interest to note very recent observations made by Dankert and Esler (39) and Tschopp et al. (37) which suggest that functional expression of the cytolytic and pore-forming activities of the C5b-9 protein complex can occur in the absence of the tubular polymerization of C9.

Although the data of the present experiments indicate that the plasma membrane Na/K-ATPase is required for membrane repolarization after C5b-9 damage, the mechanism by which target platelets restore their basal $E_m$ after complement pore formation remains unresolved. For example, repolarization of $E_m$ in the face of C5b-9 pore conductance might be achieved by a fully compensating electrogenic extrusion of cell sodium, increased turnover of the sodium pump stimulated by elevated intracellular Na$^+$. Since this mechanism implies that active Na$^+$ efflux balances the net inward conductance mediated by the C5b-9 proteins, it would require that the plasma membrane Na/K-ATPase of these cells can increase activity to exactly offset this depolarizing leak conductance. In this context it should be noted that when platelets are depolarized by thrombin stimulation (30) or by the action of added ionophores, a compensatory repolarization of the membrane potential is not observed. Alternatively, the target platelets may retain their basal electrochemical steady state by inactivating the assembled pores, for example, by shedding the C5b-9 proteins from the plasma membrane, and thereby restoring the plasma membrane to its normal resistance. Repolarization of $E_m$ would consequently follow the re-establishment of initial transmembrane ion gradients through the action of the sodium pump. An exocytotic mechanism for C5b-9 channel elimination has previously been considered in the case of nucleated cell lines in culture (11, 12). The contribution of each of these mechanisms to the reversibility of $E_m$ observed after C5b-9 binding to blood platelets is under current investigation.

In considering possible biomedical implications of the transient membrane depolarization that we observe upon binding of the C5b-9 proteins to human platelets, it is important to emphasize that the potential for a sublytic immune modulation of vascular platelet function awaits direct experimental demonstration. Furthermore, it should also be noted that other activated components of the serum complement system, including C3a, have been shown to exhibit platelet reactivity in vitro (40). Since in the present experiments platelets were exposed only to the isolated components of the membrane attack complex, our results do not permit conclusions regarding the relative importance of those changes in cell physiology mediated by the membrane-inserted C5b-9 proteins, as compared to effects likely to be mediated by other platelet-reactive components generated during activation of the complement cascades in whole plasma. Nevertheless, it should also be noted that autoimmune activation of the complement cascades normally results in only a small quantity of the C5b-9 protein to be deposited on blood cell membranes, due to regulatory control of the activation pathways by both serum and intrinsic membrane inhibitors, as well as due to the low efficiency of C5b67 binding to human cells (3). It can be suggested, therefore, that the potential for target cell activation by the C5b-9 proteins, mediated by a sublytic increase in ion permeability across the plasma membrane, is most likely to arise in the circumstance of C5b-9 binding from autologous plasma (e.g. after autoimmune activation of complement in vivo).

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