Interaction of Complement Proteins C5b-6 and C5b-7 with Phospholipid Vesicles: Effects of Phospholipid Structural Features†

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ABSTRACT: Complement components C5b-6 and C7 assemble to form C5b-7, which then interacts with membranes and commits the membrane attack complex to a target site. This protein–membrane association event was investigated to determine possible structural features that could contribute to a selective membrane attack. This system may also suggest general properties of protein–membrane insertion events. Initial binding of C5b-6 to membranes could potentially determine the site of assembly. However, binding of C5b-6 to membranes required phosphatidylglycerol or phosphatidic acid produced from egg phosphatidylcholine while binding of C5b-6 to phosphatidylcholine, phosphatidylserine, or phosphatidylinositol was undetectable. Binding to phosphatidic acid was irreversible, and the bound C5b-6 could no longer interact with C7. In contrast, C5b-7 interacted with all phospholipids tested. The rate-limiting process was the interaction of C5b-6 and C7, which displayed bimolecular properties and an activation energy of 37 kcal/mol. The C5b-7 complex showed 20-fold selectivity for small unilamellar phospholipid vesicles over large unilamellar vesicles. Vesicles carrying high negative charge densities were selected over neutral vesicles by a factor of about 5. Vesicles formed from phospholipids with short, saturated hydrocarbon side chains (dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine) were about 5-fold less effective than those formed from phospholipids with natural fatty acid distributions. The gel vs. fluid state had little influence on C5b-7 insertion. While a number of explanations for these selectivities were considered, the selectivity of C5b-7-membrane insertion, in every case, could potentially arise from the degree of exposure of the hydrocarbon portion of the bilayer membrane which interacted rapidly with an exposed hydrophobic protein segment on the C5b-7 complex. These properties may influence the specificity of attack on biological membranes. In vitro studies on assembly will be strongly influenced by the selection of membrane components.

As a result of complement activation, complement proteins C5b, C6, C7, C8 and C9 assemble on a membrane to form the membrane attack complex (MAC)[1] of complement. The MAC causes perforation of the lipid bilayer, resulting in cell damage or lysis. The scheme for MAC assembly on phospholipid bilayers (PL) is [reviewed in Muller-Eberhard (1975, 1986)]

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

The crucial step in determining the target membrane is the insertion of C5b-7 into the membrane. This step is apparently irreversible and therefore commits the MAC to a membrane site. Determination of membrane selectivity and properties of this association event should enhance general understanding of MAC assembly and may provide insight into a broad range of protein–membrane insertion events.

Several mechanisms could produce membrane selectivity. The presence of complement C3b protein on target cells enhances MAC efficiency in cell lysis, suggesting that prior binding of C5b-6 to cell-bound C3b would target MAC assembly on antigenic cells (Yamamoto et al., 1977). Hu et al. (1981) and Amiquet et al. (1985) provided indirect evidence for C5b-6 association with phospholipid membranes by cross-linking C5b-6 to photoreactive probes which were restricted to the hydrocarbon phase of phospholipid vesicles. Radiolabeled C5b-6 associated with DMPC vesicles at high vesicle/protein (300/1 w/w; Podack & Muller-Eberhard, 1978). Although C5b-6 did not bind to naked erythrocytes, treatment of the erythrocytes with an agent that induced disorder in the phospholipid bilayer resulted in significant binding of C5b-6 (Shin et al., 1981). However, there is little insight into the nature of the association of C5b-6 with membranes, the conditions required for association, or whether the association could play a significant role in MAC assembly in vivo.

The association of C5b-7 with membranes represents a novel system for study of a protein–membrane association event. The C5b-7 complex associates with the bilayer with properties suggesting an integral membrane protein [e.g., see Podack et al. (1981) and Stewart et al. (1984)]. However, unlike other systems of integral membrane proteins which require detergent treatment or reconstitution techniques to acquire inserted protein, the C5b-7 complex can be formed from soluble C5b-6 and C7 in the presence of membranes, and the insertion event can be studied in a mixture of soluble monomeric proteins and phospholipid vesicles. However, the absolute kinetics of the association event cannot be directly monitored since the rate-limiting step is the relatively slow association of C5b-6 with C7 (Podack et al., 1978a). Properties of the membrane

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[1] Abbreviations: MAC, membrane attack complex; PL, phospholipid; PC, phosphatidylcholine from egg; PS, phosphatidylserine isolated from bovine brain; PG, phosphatidylglycerol; PA, phosphatidic acid produced from egg PC; PI, phosphatidylinositol; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicle (diameter about 30 nm); LUV, large unilamellar vesicle (diameter about 100 nm or greater); 6CF, 5(6)-carboxyfluorescein; Tris, tris(hydroxymethyl)aminomethane.

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association event must therefore be determined by indirect techniques.

The actual protein component that is involved in C5b-7-membrane contact appears to be C7. Recent evidence shows that C7 undergoes a conformation change and aggregation by itself when exposed to detergents or denaturants (Preissner et al., 1985a,b). It is possible that this change in C7 is analogous to one induced by C5b-6, which exposed a hydrophobic membrane-association site on the C7 molecule.

The studies reported here were initiated to investigate C5b-7 insertion into model membrane structures and determine the membrane properties that influence this reaction. The results gave insight into the general characteristics of this protein–phospholipid association, suggesting a probable rate-limiting event consisting of protein penetration through the head group region of the membrane. The results showed possible mechanisms for generating membrane selectivity and showed important considerations for in vitro studies of MAC assembly.

Materials and Methods

Proteins. Complement components C5b-6 (Silversmith & Nelsestuen, 1986a), C7 (Podack et al., 1979b), C8 (Steckel et al., 1980), and C9 (Biesecker & Muller-Eberhard, 1980) were purified from human plasma as described.

Vesicles. All vesicles were prepared with highly purified phospholipids. PC (egg yolk), PI (soybean), PS (bovine brain), PG (derivative of egg yolk PC), PA (derivative of egg yolk PC), and DOPC (synthetic) were purchased from the Sigma Chemical Co., St. Louis, MO. DMPC and DPPC were purchased from Avanti Polar Lipids, Birmingham, AL.

Small unilamellar vesicles with 200 mM 5(6)-carboxyfluorescein (6CF) trapped in the interior were prepared by sonication and gel filtration on Sepharose 4B as described (Silversmith & Nelsestuen, 1986a).

Vesicles without the fluorescent probe were prepared by three different methods to obtain vesicle populations with a variety of sizes and phospholipid compositions. For phospholipids that were soluble in ethanol (PG, PC, DMPC, DOPC, and DPPC), small unilamellar vesicles were prepared by the method of ethanol injection (Kremer et al., 1977). Briefly, 1 mg of phospholipid was dried under a stream of N_2, dissolved in 0.5 mL of absolute ethanol, and injected in 5 μL portions into stirring Tris buffer at 25 °C. For vesicles composed of DPPC, the Tris buffer was warmed to 55 °C during injection (above the fluid to solid phase transition temperature of DPPC, 41 °C).

Small unilamellar vesicles were also prepared by sonication according to the method of Huang et al. (1969). Phospholipids (10–40 mg) were dried under a stream of N_2 and resuspended in 3.5 mL of Tris buffer by vigorous agitation. The suspension was sonicated by using a direct probe for six 30-s treatments at 150 W of power. The sample was kept cool with an ice bath during sonication. The sonicated sample was gel filtered on a Sepharose 4B column (1.5 × 25 cm). A single fraction (2 mL) from the column elution was used to obtain a monodisperse vesicle population.

Large unilamellar vesicles were prepared by extrusion (Hope et al., 1985). Phospholipid (5–10 mg) was dried under a stream of N_2 and resuspended in 3 mL of Tris buffer by vigorous agitation. The suspension was freeze/thawed 5 times by using dry ice/alcohol and 37 °C water baths and then forced through a 0.1-μm filter (Nucleopore Corp., Pleasonton, CA) 10–15 times by using an Amicon ultrafiltration cell (30 psi of N_2). The vesicles prepared by this method were analyzed by dynamic light scattering using a Langley/Ford LSA2 light scattering photometer. The vesicles had a high degree of homodispersity with a Z-averaged hydrodynamic diameter that ranged from 95 to 110 nm for vesicle preparations of various phospholipid compositions.

Very large unilamellar vesicles were prepared by the method of ether injection (Deamer & Bangham, 1976) followed by extrusion. PC (20 mg) dissolved in 3 mL of diethyl ether was injected into 5 mL of buffer which was warmed to 60 °C. This was followed by extrusion of the solution through a 0.600-μm polycarbonate filter under low N_2 pressure. The resulting vesicles had a Z-averaged diameter of 500 ± 100 nm as determined by dynamic light scattering. The range given was that obtained when different machine time constants were used for estimation of particle diffusion constants. This change with machine setting indicated a heterogeneous population of vesicles, and the true average diameter was not well-known. Similar results have been observed by others (Mayer et al., 1986) for very large vesicles (>200 nm) produced by extrusion techniques. The diameter measured by electron microscopy was also heterogeneous and slightly smaller than the pore size of the membrane (Mayer et al., 1986). In any event, the actual average diameter of the vesicles produced by this technique was several times larger than 100 nm, and the preparations can be used qualitatively in experiments designed to be sensitive to differences in vesicle size. These vesicles are referred to in the text as 500-nm vesicles.

Phospholipid concentration was determined by assay of organic phosphate (Chen et al., 1956). The phospholipid/phosphorous ratio used in the calculations was 25 for all biological phospholipids, 22.6 for DMPC, 24.5 for DPPC, and 25.4 for DOPC.

Fluorescence Measurements. The fluorescence intensity of 5(6)-carboxyfluorescein-containing vesicles was used to monitor the binding of C5b-8 to vesicles. The vesicles alone had a relatively low fluorescence intensity due to the self-quenching of 5(6)-carboxyfluorescein at the high (200 mM) concentration inside the vesicles. Addition of Triton X-100 (final concentration of 1%) caused lysis of the vesicles and a 15–20-fold increase of fluorescence intensity due to dilution of the probe into solution. Addition of C5b-6 did not affect the fluorescence intensity of the intact vesicles. Formation of C5b-7 caused at most a very slow intensity increase. Subsequent addition of C8 to form C5b-8 caused a rapid increase in fluorescence intensity (ΔF) due to lysis of the vesicles. Addition of C9 caused no further change in fluorescence intensity. The relationship between the molar ratio of C5b-8/vesicle and the percent vesicle lysis has been shown to correlate with that predicted for statistically random binding of C5b-7, with one C5b-8 complex per vesicle adequate for lysis (Silversmith & Nelsestuen, 1986a). Maximal lysis at saturating levels of C5b-8 gave about 70–80% of the fluorescence intensity change obtained in the presence of Triton. Similar characteristics were observed for 6CF-containing vesicles composed of either 100% PC or 100% PG.

The fluorescence intensity of 5(6)-carboxyfluorescein was measured with excitation at 497 nm (4-nm slit width) and emission at 518 nm (8-nm slit width) on a Perkin-Elmer 44A spectrofluorometer. All experiments were done in 1.5 mL of Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4) Generally, 1.5-2.0 μg of vesicles were used in the assay.

The quantitity of C5b-7 bound to the 6CF-containing vesicles in the presence and absence of a second population of vesicles was determined by the extent of 6CF release (measured by fluorescence increase) after C8 addition. A quantity of C5b-6 that gave 30–35% maximal lysis (~1 C5b-8/3 6CF-containing vesicles) was used as the limiting

component in the competition assays. At this protein/lipid ratio, the relationship between the C5b-8/vesicle ratio and 6CF released was approximately linear. A decrease in fluorescence due to C5b-7 association with the competing lipid was proportional to the quantity of C5b-8 bound to the competing population. A constant amount of 6CF-containing vesicles and various amounts of the competing lipids were mixed with the appropriate quantity of C5b-6. Excess C7 was added and 1–2 min was allowed for C5b-7 assembly. Finally, excess C8 was added. The percent of total C5b-8 associated with the 6CF-containing vesicles was determined from the simple relationship:

$$(F/F_0) \times 100 = \text{percent C5b-8 associated with 6CF vesicles} \quad (1)$$

where F_0 is the change in fluorescence after C8 addition to the 6CF-containing vesicles without a second vesicle population and F is the fluorescence change in the presence of the second vesicle population.

Light Scattering. Light scattering intensity measurements at 90° were used to monitor protein binding to phospholipid vesicles and fluid-phase assembly of C5b-7. The theory and methods (Nelsestuen & Lim, 1977) and their application to study of the membrane attack complex (Silversmith & Nelsestuen, 1986a,b) have been described in greater detail.

For this protein–vesicle interaction, the ratio of the molecular weight or weight concentration of the protein–vesicle complex ($M_{r,2}$ or c_2) to the molecular weight or weight concentration of the vesicles ($M_{r,1}$ or c_1) can be estimated from

$$(c_2/c_1)^2 = (M_{r,2}/M_{r,1})^2 = (I_{s,2}/I_{s,1})/[(\partial n/\partial c_1)/(\partial n/\partial c_2)]^2 \quad (2)$$

$I_{s,1}$ is the light scattering intensity of the vesicles, and $I_{s,2}$ is the light scattering intensity after addition of protein. $\partial n/\partial c$ are the refractive index increments (0.19 for pure protein and 0.17 for phospholipid vesicles). The $\partial n/\partial c$ of the protein vesicle complex was a weighted average.

For fluid phase assembly, eq 3 was applied, where I_s is the net light scattering intensity of the assembled $(C5b\text{-}7)_n$, I_{benz}

$$M_r = I_s/[I_{benz}k(\partial n/\partial c)^2 c] \quad (3)$$

is the light scattering intensity from benzene that is standardized to known samples by the constant k, and c is the weight concentration of light scattering material. Both eq 2 and eq 3 give weight concentrations of product. These were converted to molar concentrations by using a molecular weight for the fluid-phase aggregate, $(C5b\text{-}7)_n$, of 4.1×10^6 and a molecular weight for a single C5b-7 complex of 445 000 (Silversmith & Nelsestuen, 1986b). Equation 3 was also used to estimate the molecular weight of small unilamellar vesicles.

For time-dependent analyses, known amounts of C5b-6 or C7 were placed in the cuvette (with or without phospholipid), and the second protein was added at zero time. The extent of assembly was estimated at various times from eq 2 or 3. Under the conditions of these experiments, the reaction was second order; the rate of C5b-6 assembly with C7 was rate-limiting, and subsequent assembly of C5b-7 on the membrane or fluid-phase aggregation was rapid [see below and also Podack et al. (1978a)]. When C7 and C5b-6 were present in equal quantity, second-order rate constants were obtained from the slope of $1/[C7]$ plotted vs. time. When these proteins were present in unequal quantity, the second-order rate constants were obtained from the slope of $[1/([R_1] - [R_2])]$ ln $[(1 - [P]/[R_1])/(1-[P][R_2])]$ plotted vs time. In the latter relationship, P is product, R_1 is the most abundant protein, and R_2 is the limiting protein. The 100% reaction level was

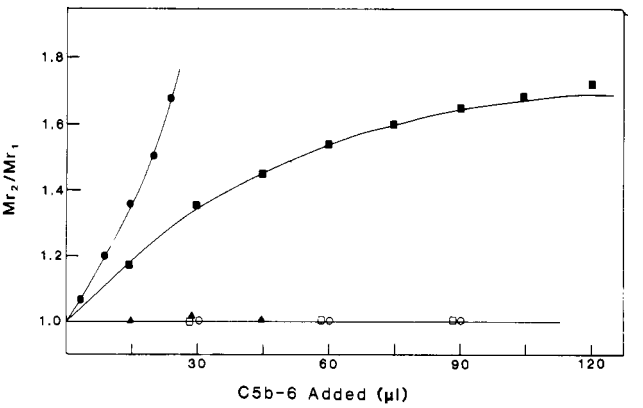

FIGURE 1: Interaction of C5b-6 with SUVs with various polar head groups. C5b-6 (110 μg/mL) was added in sequential aliquots to vesicles and the light scattering intensity recorded after each addition. The $M_{r,2}/M_{r,1}$ ratio, where $M_{r,2}$ is the molecular weight of the vesicles plus bound protein and $M_{r,1}$ is the vesicle weight, was calculated by using eq 2. The vesicle compositions were 100% PA (40 μg, ●), 100% PG (20 μg ■), 100% PC (same result for 0.2–20 μg, ○); 100% PS (same result for 0.2–20 μg, □), and 100% PI (10 μg, ▲).

obtained after incubation of the sample at room temperature for 5 min followed by cooling to the temperature indicated. The rate constants were obtained from data collected between 0% and 60% of completion. This portion of the curve gave linear plots with correlation coefficients of >0.99. The last 25% of the reaction often showed downward curvature. The latter may have been due to any of several factors that were not investigated. For example, minor variations in assigning the 100% reaction point might be responsible for some curvature.

The Z-averaged diffusion coefficients and hydrodynamic particle sizes of the vesicle preparations were determined by dynamic light scattering as described previously (Silversmith & Nelsestuen, 1986a,b) except that the instrument used was a Langley/Ford LSA2 particle sizing apparatus equipped with a Langley/Ford 1096 autocorrelator.

Unless indicated, all experiments were conducted in 0.05 M Tris buffer, pH 7.5, containing 0.1 M NaCl.

RESULTS

Association of C5b-6 with SUV. The association of C5b-6 with various vesicle populations was examined by using light scattering intensity measurements (Figure 1). No excess light scattering intensity was observed when C5b-6 was added to vesicles composed of PS, PC, or PI. However, there was binding of C5b-6 to vesicles composed of PA and PG. For PG vesicles, addition of C5b-6 resulted in rapid, stable changes in light scattering intensity until saturation was achieved. The $M_{r,2}/M_{r,1}$ values from the intensity increases (Figure 1) were about twice the theoretical value for quantitative binding of the added C5b-6 to the vesicles. This suggested that C5b-6 induced some aggregation of the PG vesicles. The first additions of C5b-6 to PA vesicles also caused rapid, stable increases in light scattering intensity. However, the changes far exceeded the theoretical value for quantitative binding of the added C5b-6 to monomeric vesicles, indicating that C5b-6 also induced aggregation of PA vesicles. Larger addition of C5b-6 to PA vesicles caused large, time-dependent intensity changes, indicating extensive aggregation, and the light scattering signal could not be saturated.

Addition of C7, C8, and C9 to PA-bound C5b-6 caused very small intensity changes compared to those that occurred after adding the same quantities of these proteins to C5b-6 in the absence of vesicles (data not shown). It appeared that PA-

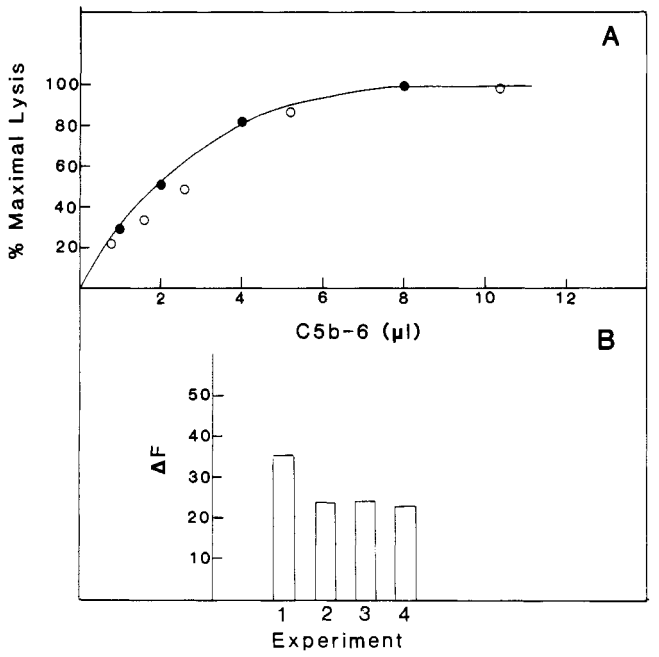

FIGURE 2: Lysis of 100% PG vesicles containing 6CF by C5b-8. (A) Titration of vesicles containing 6CF with C5b-6. Aliquots of C5b-6 (0.12 mg/mL) were added to PG vesicles (4.4 μg, O) as the limiting component, followed by excess C7 and C8. The C8-dependent fluorescence change was measured and plotted as a percentage of the fluorescent change when C5b-8 was added in saturating quantity (ΔF_{max}). Also shown is an identical titration for an equimolar quantity of 6CF-containing PC vesicles with C5b-6 (●). (B) The quantity of C5b-8 used corresponded to 33% maximal lysis of the 6CF-containing PG vesicles (4.4 μg in 1.5 mL). Nonfluorescent PG (3.0 μg) was used as a competing phospholipid. The total fluorescence change upon protein assembly (ΔF) is shown for the following experiments: (*1*) C5b-6 was added to 6CF-containing vesicles in the absence of another population of vesicles. (2) C5b-6 was added to the mixture of vesicle populations followed by C7 and C8. (3) C5b-6 was added to the nonfluorescent vesicles, followed by addition of the fluorescent vesicles, C7, and C8. The second phospholipid and C7 were added within 10 s of each other for experiments 3 and 4. All experiments were in total volume of 1.5 mL.

bound C5b-6 was unreactive and that it did not dissociate from the PA vesicles to form fluid-phase C5b-7. Addition of C7, C8, and C9 to PG-bound C5b-6 resulted in excess light scattering intensity which was about double the increase for fluid-phase assembly. This result is consistent with assembly of C5b-7 on the vesicles (Silversmith & Nelsestuen, 1986b) although quantitation was not possible due to the prior vesicle aggregation.

Titration of PG vesicles containing 6CF with C5b-8 showed that C5b-7 bound very efficiently (Figure 2A) since approximately equal quantities of C5b-8 were required to lyse equimolar quantities of PG and PC vesicles. Previous studies (Silversmith & Nelsestuen, 1986a) showed that, under the conditions of these titrations, C5b-7 bound quantitatively to the PC vesicles.

Binding of C5b-6 to one population of vesicles before addition of C7 did not enhance the membrane targeting of MAC assembly. The distribution of C5b-7 over two populations of PG vesicles (a nonfluorescent population and a population containing 6CF) was independent of whether C5b-6 was initially added to the fluorescent vesicles, the nonfluorescent vesicles, or a mixture of the two populations (Figure 2B). Because C5b-7 did not dissociate significantly from the membrane (see below), rapid equilibration of C5b-6 over the two populations had to occur before reaction with C7. This would be a natural consequence if C7 reacted exclusively with free C5b-6. The latter property would also explain the unreactivity of PA-bound C5b-6.

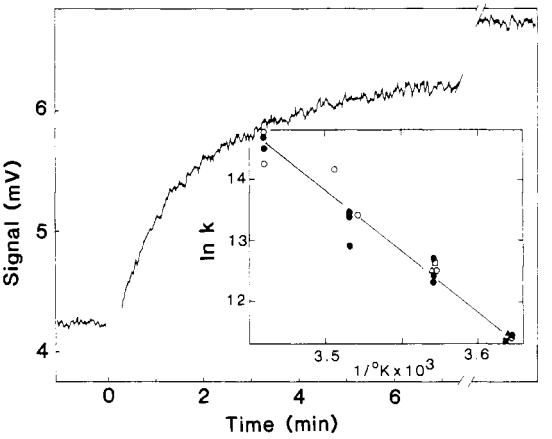

FIGURE 3: Assembly of C5b-7 assayed by light scattering. Phospholipid vesicles (PC, 90 μg in 1.5 mL) gave the light scattering intensity shown at 7 °C. Proteins C5b-6 (17 μg) and C7 (6.3 μg) were added at zero time and intensity changes monitored continuously. Second-order rate plots of the data were constructed by using light scattering intensity to calculate the concentration of product at different times (see Materials and Methods), and rate constants were plotted (inset; units are M^{-1} s^{-1}) for temperatures from 3 to 16 °C. Different conditions used in the inset include the following: assembly on the membrane at the protein concentrations given (●), fluid-phase assembly using the same amounts of the proteins (O), membrane assembly (▲) and fluid-phase assembly (△) at 8.5 μg of C5b-6 and 6.3 μg of C7, and membrane assembly (■) and fluid-phase assembly (□) at 17 μg of C5b-6 and 3.1 μg of C7. The activation energy indicated from the line drawn is 37 kcal/mol.

Assembly of C5b-7. Podack et al. (1978a) reported that interaction of C5b-6 with C7 is the rate-limiting step and subsequent fluid-phase aggregation or membrane binding are rapid at low temperature. This was confirmed here by observations of similar second-order rate constants with varying C7 and C5b-6 concentrations (Figure 3, inset). Formation of C5b-7 can be directly monitored by light scattering changes and the kinetics of the reaction were readily observed at low temperature (Figure 3). The rate constants for the C5b-7 reaction were estimated from the light scattering intensity changes as outlined under Materials and Methods and are presented in Figure 3 (inset) as an Arrhenius plot. The activation energy for this reaction was determined to be 37 kcal/mol, which is very similar to the value of 33.7 obtained by Podack et al. (1978a). However, the absolute values for the rate constants shown in Figure 3 were about 10-fold higher than those obtained by Podack et al. (1978a). The basis for this difference is not known. However, the lower rate constants reported by Podack et al. (1978a) would give readily observable rates for C5b-7 assembly even at 20 °C. This was not observed (Silversmith & Nelsestuen, 1986a).

Dissociation of C5b-7 from Vesicles. Possible dissociation of C5b-7 from vesicles was studied by determining the loss of C5b-7 from one vesicle population as a function of time after addition of a large excess of another lipid population. C5b-7 was first assembled on vesicles containing 6CF. An added population of excess nonfluorescent vesicles would trap any C5b-7 that dissociated, and release of 6CF (F) upon subsequent addition of C8 would be reduced. Figure 4 shows that there was less than a 35% decrease in 6CF release after incubation of these mixtures for a 2-h period. The result was the same for C5b-7 assembled on vesicles composed of PC or PG. Since a similar loss of 6CF release was observed in the absence of a second vesicle population (buffer control, Figure 4), the decrease in fluorescence change could not be attributed to dissociation of functional C5b-7 from the fluorescent vesicles

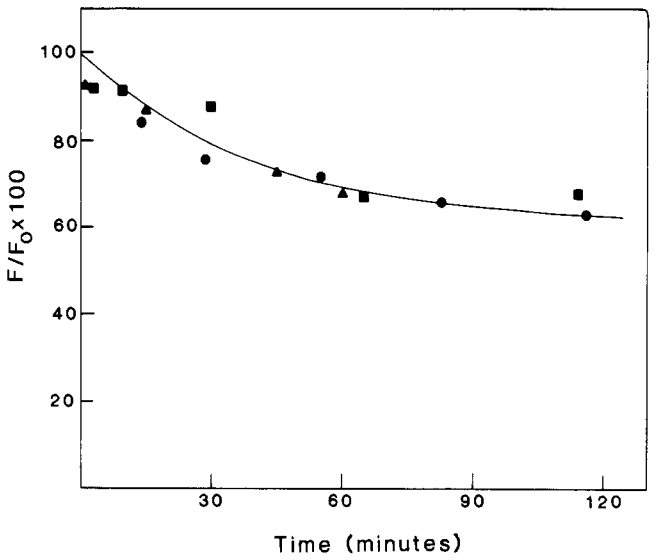

FIGURE 4: Dissociation of C5b-7 from vesicles. C5b-7 was assembled on 6CF-containing vesicles of 100% PC (10.8 μg, ● and ▲) or 100% PG (28.6 μg, ■) (C5b-7/vesicle = 0.3) in a final volume of 9 mL. At zero time, 0.6 mL of nonfluorescent PC (85 μg, ●), buffer (▲), or nonfluorescent PG (180μg, ■) was added. At various time points, aliquots (1.5 mL) were removed, and the fluorescence change after C8 addition was measured (F). F_0 was the fluorescence change after addition of excess C8 to 1.5 mL of the mixture at zero time and before addition of the second vesicle population.

and reassociation with the nonfluorescent vesicles. Loss of fluorescence change could be due to time-dependent inactivation of C5b-7 on the membrane or inability of dissociated C5b-7 to reassociate with the nonfluorescent vesicles. In any event, the results indicated a maximum dissociation rate constant of 6.2×10^{-5} s^{-1} for the C5b-7–vesicle complex. This rate is negligible on the time scale of subsequent experiments so the distribution of C5b-7 over two vesicle populations would be a function of the relative rate of association of C5b-7 with the various lipids and not a function of equilibrium binding to the various lipids.

Effect of Polar Head Group in C5b-7 Association with Vesicles. Figure 5A shows the ability of SUVs with various polar head groups to inhibit the binding of C5b-7 to PC vesicles containing 6CF. Small vesicles composed of PC (prepared by ethanol injection) gave a linear slope of −1, indicating that the C5b-7 complex was distributed randomly over the two vesicle populations. This result demonstrated that SUVs prepared by ethanol injection and sonication were indistinguishable to C5b-7 and that 6CF trapped in the interior of vesicles did not affect the association of C5b-7.

Vesicles composed of 100% PG, PS, and PI gave competition curves showing that, on a weight basis, these vesicles were more efficient than PC in binding C5b-7. Relative rates for PG, PS, and PI were as follows: $V_{PG}/V_{PC} = 6.7$, $V_{PS}/V_{PC} = 4.5$, and $V_{PI}/V_{PC} = 2.5$ (Table I), respectively. The relative velocities were calculated from the C5b-7 distribution when the two vesicle populations were present in equal weight quantity.

That PG and PS showed similar behavior was consistent with the proposal (above) that C5b-6 bound to PG vesicles rapidly dissociated and subsequently bound C7. If C7 associated directly with PG-bound C5b-6, PG should be much more effective than PS in binding C5b-7 since PS did not bind C5b-6.

Vesicles composed of PA were extremely efficient in inhibiting the association of C5b-7 with the PC vesicles containing 6CF. Unlike the other vesicles examined, the competition was linear with PA concentration until total inhibition

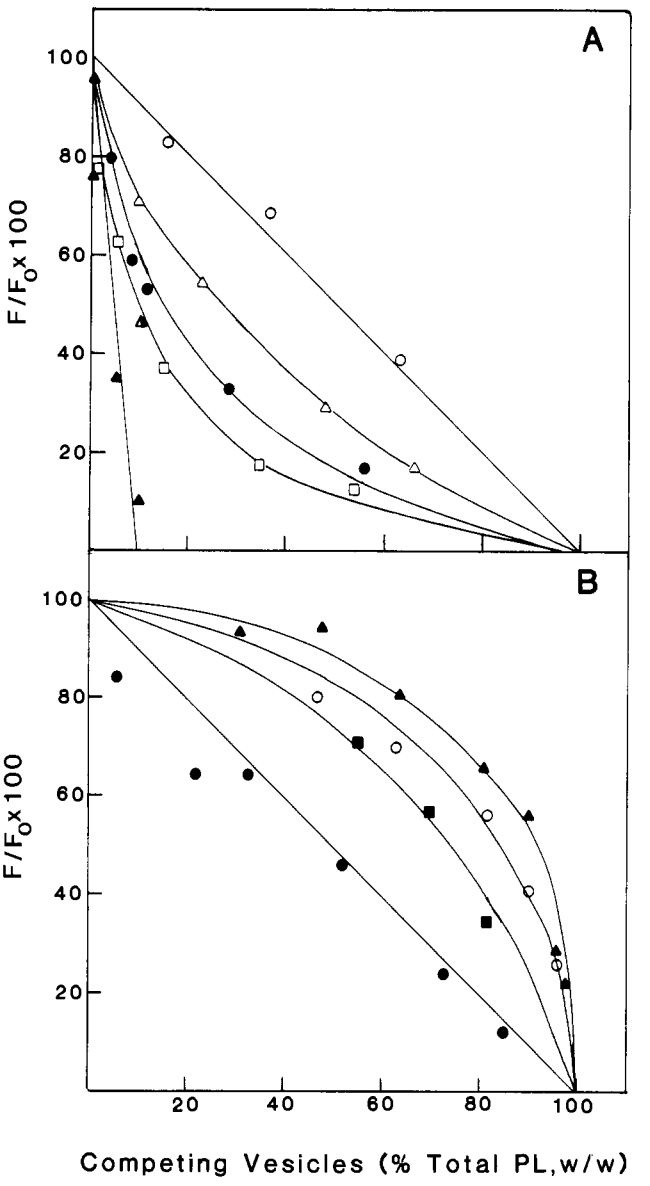

FIGURE 5: Competition between 6CF-containing PC vesicles and nonfluorescent vesicles for C5b-7 binding. The experiments were described under Materials and Methods. (A) The competitive vesicle populations were all SUVs and consisted of 100% PC (○), 100% PI (△), 100% PS (●), 100% PG (□), and 100% PA (▲). Also shown is the result of addition of C5b-6 to PA vesicles that were already mixed with a 3-fold excess of C7 (▲). (B) The competing vesicle populations were all SUVs and consisted of 100% DMPC, 17 °C (○), 100% DMPC, 32 °C (▲), 100% DPPC, 25 °C (■), and 100% DOPC, 25 °C (●). The ratio of fluorescence intensity for the experiment shown (F) is expressed relative to the intensity (F_0) without a second phospholipid population present.

was achieved (Figure 5A). The behavior was consistent with irreversible binding of C5b-6 to the vesicles. In further support of this conclusion, addition of C5b-6 as the final component to a mixture containing excess C7, PA, and 6CF PC vesicles dramatically reduced the degree of competition by PA (Figure 5A). The presence of excess C7 would allow fluid-phase formation of C5b-7, which could then distribute among the PC and PA vesicles.

Influence of Side Chain and Phase State of Bilayer. The side chains of egg yolk PC consist almost entirely of palmitic and oleic acids (1:1). Figure 5B shows the ability of SUVs composed of 100% DMPC, 100% DPPC, and 100% DOPC to compete with egg yolk PC for association of C5b-7. The 1:1 competition by DOPC vesicles indicated that C5b-7 bound DOPC and egg yolk PC with equa rates ($V_{DOPC}/V_{PC} = 1$,

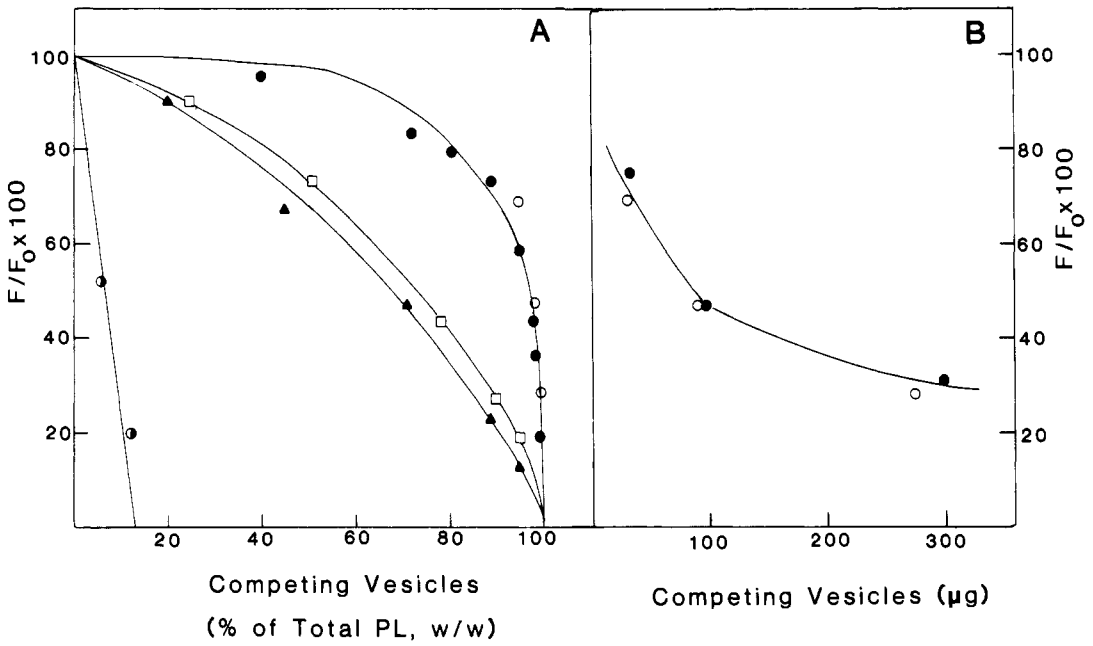

FIGURE 6: Competition between vesicles of different size for C5b-7. The experiments were similar to those described in Figure 5. The vesicle populations were 100% PC (100-nm diameters, ●). 100% PC (500 nm, ○), 100% PS (100 nm, □), 100% PG (100 nm, ▲), and 100% PA (100 nm, ◑). Panel A shows the data plotted as in Figure 5A. Panel B shows the data plotted as a function of the absolute quantity of the second phospholipid present for PC vesicles of 100 nm (○) and 500 nm (●).

Table I: Selectivity of C5b-7 for Various Vesicle Populations

vesicle type	method of preparation	hydrodynamic diameter[a] (nm)	V_{PL}/V_{PC}[b]
PC SUVs	ethanol injection	35	1
PG SUVs	ethanol injection	22	17
DMPC SUVs	ethanol injection	31	0.20 (17 °C)
DMPC SUVs	ethanol injection	31	0.15 (32 °C)
DPPC SUVs	ethanol injection	45	0.32
DOPC SUVs	ethanol injection	51	1.0
PG SUVs	sonication and gel filtration	31	6.7
PS SUVs	sonication and gel filtration	38	4.5
PI SUVs	sonication and gel filtration	38	2.5
PC LUVs	extrusion	112	0.05
PC LUVs	extrusion	500 ± 100	0.05
DMPC LUVs	extrusion	97	0.015
PG LUVs	extrusion	99	0.47
PS LUVs	extrusion	95	0.33

[a] The diameter of LUVs was measured by dynamic light scattering. For SUVs, the diameter was calculated from the molecular weight determined by mass concentration and light scattering intensity of the phospholipid (see Materials and Methods); the volume of lipid was equal to the volume of a hollow sphere with bilayer thickness equal to 4 nm and a density of 1.0 g/cm^3. [b] The ratio of velocities of association of C5b-7 with the lipid compared to association with PC SUVs. This value was obtained from the C5b-7 distribution when the lipid weight concentrations were equal. Unless indicated, all experiments were conducted at 25 °C.

Table I). Vesicles with shorter side chains were less efficient than egg yolk PC (V_{DPPC}/V_{PC} = 0.32, V_{DMPC}/V_{PC} = 0.20, Table I). The decreased efficiency of C5b-7 binding to DMPC was not due to dissociation of C5b-7 from DMPC vesicles; prolonged incubation (1 h) of the C5b-6 and C7 with the mixture of DMPC and PC did not change the amount of 6CF release upon C8 addition. Reversible association with DMPC would result in greater accumulation of C5b-7 on the 6CF-containing PC vesicles and increased release of 6CF after this incubation. The results indicated that vesicles composed of the shorter chain phospholipids had structural features that made membrane insertion of C5b-7 a less efficient process.

Similar competition curves were observed for DMPC vesicles below (17 °C; V_{DMPC}/V_{PC} = 0.2) and above (32 °C; V_{DMPC}/V_{PC} = 0.15, Table I) the phase transition temperature of DMPC (23 °C). Therefore, the rigidity of the bilayer had little influence on the rate of C5b-7 association with the vesicles.

Effect of Vesicle Size. Figure 6 shows the ability of LUVs of various phospholipid compositions to compete for C5b-7 binding with SUVs containing 6CF. The velocity of C5b-7 association with LUVs of PC was about 20 times slower than with an equal weight quantity of SUV (V_{LUV}/V_{SUV} = 0.05, Table I). Similarly, LUVs composed of PS or PG were much less efficient than SUVs of the same phospholipid. However, C5b-7 bound to LUVs composed of PG and PS about 7- and 5-fold more efficiently, respectively, than to LUVs of PC. Therefore, enhanced binding of C5b-7 to negatively charged bilayers also occurred with the large vesicles. However, the low binding efficiencies for the LUVs indicated that vesicle size strongly influenced association of C5b-7. This trend did not continue for very large unilamellar vesicles ($2R_h$ = 500 nm), which were comparable to 100 nm LUVs on a weight basis (Figure 6B). LUVs of PA showed properties of irreversible C5b-6 binding as described for SUVs of PA.

C5b-7 Binding to Vesicle Populations Assessed by Light Scattering. The C5b-7 complex aggregates in the absence of membranes (Silversmith & Nelsestuen, 1986a). Increasing concentrations of vesicles in the presence of a constant amount of C5b-7 increased the proportion of the C5b-7 bound to vesicles until quantitative binding is achieved (Silversmith & Nelsestuen, 1986b). The results showed that some vesicle populations (e.g., PG and PS) bound C5b-7 more efficiently than PC so that lower concentrations of these vesicles should be required for quantitative C5b-7 binding. Likewise, higher concentrations of vesicles that bound C5b-7 less efficiently than PC (e.g., DMPC) should be necessary to achieve quantitative binding of the same quantity of C5b-7.

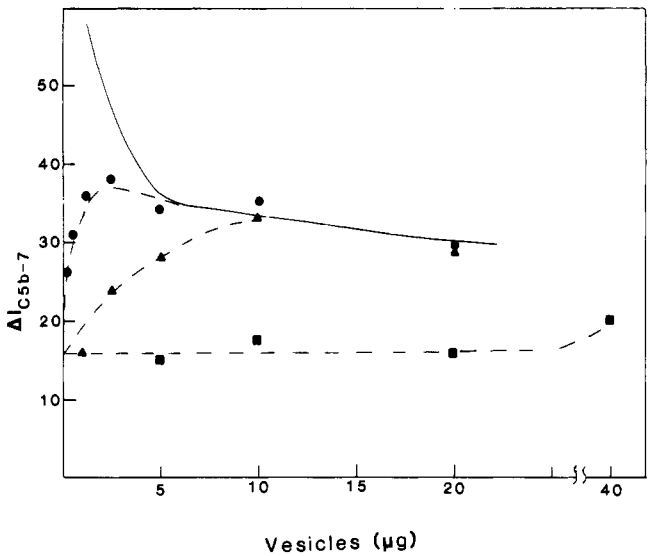

FIGURE 7: Light scattering intensity after addition of C5b-7 to various populations. C7 (3.3 μg) was added to a mixture of C5b-6 (2.6 μg) and vesicles consisting of 100% PS (●), 100% PC (▲), or 100% DMPC (■) in a total volume of 1.5 mL. The relative intensity increase after addition of C7 (ΔI_{C5b-7}) is plotted. The value for zero phospholipid is due to the fluid-phase aggregation of C5b-7. The dashed lines connected the data points, and the solid line is the predicted theoretical values for quantitative binding of C5b-7 at all vesicles concentrations.

Light scattering measurements corroborated this finding. Addition of C7 to C5b-6 in the presence of PS or PC vesicles caused larger intensity changes than those obtained for fluid-phase assembly (Figure 7). The ΔI_{C5b-7} observed for PS (>5 μg/mL) and PC (>10 μg/mL) equaled the theoretical values for quantitative binding of the C5b-7 added (3.1 μg) to the vesicles (Figure 7). Below these lipid concentrations the intensity changes were less than theoretical, indicating significant fluid-phase assembly. The DMPC vesicles did not appear to bind all of the protein even at the highest DMPC concentrations. The theoretical ΔI value increases at low lipid concentrations since association of many proteins with a few vesicles produces larger changes than association of the same amount of protein with many vesicles (Pusey & Nelsestuen, 1984). Both light scattering and competition studies showed that C5b-7 associated preferentially with phospholipids in the sequence PS > PC > DMPC.

Discussion

Complement component C5b-6 is a stable, water-soluble complex that is generally thought not to interact directly with target membranes during MAC assembly. Although there have been reports (Hu et al., 1981; Amiguet et al., 1985) that C5b-6 would associate with some synthetic phospholipid vesicles, these studies used indirect methods such as chemical cross-linking, and measurements were made on only one type of vesicle. Our results showed that C5b-6 did bind to small unilamellar vesicles composed of 100% PG and 100% PA but not to vesicles composed of 100% PS, PI, or PC. The association of C5b-6 with both PG and PA vesicles induced vesicle–vesicle association. Whether the ability of C5b-6 to associate with negatively charged vesicles has any relevance to MAC assembly in vivo is not known but seems unlikely. The results suggested that neither PA- nor PG-bound C5b-6 would directly bind C7, a result that would eliminate the influence of initial C5b-6 binding in determining the target membrane. However, C5b-6 may be involved in determining the membrane attack site through protein–protein interactions; the reported binding of C5b-6 to C3b on the target membrane may be a mechanism of producing selective attack of the MAC on an antigenic cell (Hammer et al., 1976; Yamamoto et al., 1977).

Dissociation of C5b-7 from vesicles was extremely slow ($k_d \leq 6.2 \times 10^{-5}$ s^{-1}), consistent with an integral membrane protein. Other well-characterized integral membrane proteins also have slow dissociation rates [e.g., cytochrome b_5 (Enoch et al., 1970) and b_5 reductase (Leto et al, 1980)]. On the other hand, peripheral phospholipid-binding proteins such as blood coagulation factors V (Pusey et al., 1982) and prothrombin (Wei et al., 1982) dissociate from vesicles in milliseconds to minutes.

PC-containing vesicles of different sizes showed very different efficiencies of C5b-7 addition as indicated by competition experiments where lipid was expressed on the basis of weight concentration; larger vesicles were less effective in binding C5b-7 than small vesicles. This observation will be important in designing and interpreting experiments that utilize phospholipid vesicles of different or heterogeneous size. It may also suggest important properties related to the C5b-7–membrane interaction process. A number of factors can be considered.

First of all, SUVs have about two-thirds of their lipid on the exterior of the vesicle while LUVs have only half of the lipid on their exterior; SUVs should show about 30% greater effectiveness per unit mass. This accounts for only a small portion of the 20-fold differences observed.

Second, a factor relating to translation diffusion parameters can be considered. For a bimolecular reaction such as the interaction of C5b-7 with phospholipid, the velocity will be given by the second-order expression

$$v = k[\text{C5b-7}][\text{L}] \quad (4)$$

where L is lipid. If the reaction is 100% collisionally efficient, a case that is approached for a number of extrinsic protein–membrane binding events (Pusey & Nelsestuen, 1984), the correct term to use for lipid will be the molar concentration of particles (vesicles). If the reaction is substantially less than 100% collisionally inefficient, the correct term will be the molar or weight concentration of phospholipid molecules needed to bind one C5b-7 complex. This difference is related to collisional processes in solution. The rate constant for collision of two particles in solution is described by the theory of Schmoluchowski (1915, 1917):

$$k = 4\pi N_0 Da/1000 \quad (5)$$

where a is the sum of the radii of the two colliding particles (for many cases this approximates the radius of the phospholipid vesicles since the proteins are substantially smaller) and D is the sum of the diffusion constants of the particles (this approaches the diffusion constant of the protein). The molar concentration of particles is proportional to the weight concentration of lipid divided by the molecular weight of the vesicle. The latter will be proportional to $4\pi a^2$ when a constant 50% of the phospholipids are on the exterior of the vesicle. Use of molar concentration of vesicles in eq 4 therefore provides that velocity at a constant weight concentration of phospholipid will be approximately proportional to a/a^2, and phospholipids dispersed in smaller vesicles will give higher velocity than phospholipids dispersed in large vesicles.

Increased efficacy of small vesicles on a weight basis was observed in this study. However, this trend was not continued to the 500-nm vesicles, which were about equally effective on a weight basis as the 100-nm vesicles. Consequently, interaction of C5b-7 with PC vesicles of 100- and 500-nm diameter did not correlate with the anticipated properties of a colli-

sionally efficient reaction. That this conclusion also applies to the SUVs of PC was indicated by the observation that SUVs of PG and PS were 5–15 times more effective than SUVs of PC; in the absence of long-range interactions, vesicles of similar size should give similar collisional rate constants.

For a reaction that is substantially less than collisionally efficient, diffusional equilibrium is achieved for the interacting particles and the number of collisions between a protein and phospholipid vesicle is proportional to the collisional rate constant for macrocollisions times a term for the number of microcollisions between two particles before they escape to infinite distance. The latter term is also proportional to a (Berg & Purcell, 1977). The translational diffusional component of the association rate constant will be proportional to a^2, and the overall velocity of interaction of C5b-7 with a given weight concentration of phospholipid will be unaffected by vesicle size (except for unequal phospholipid distributions on the exterior of the different vesicle populations). The results obtained here suggested that interaction of C5b-7 with PC vesicles of all sizes was considerably below the collisional rate so that translational diffusion did not explain the differences between SUV and LUV lipids.

Third, rotational diffusion of the vesicles could also increase the efficiency of SUVs. If C5b-7 requires a specific structure formed on the membrane bilayer such as a transient perturbation in head group packing, factors that increase the probability of finding this structure will enhance C5b-7 binding. Reorientation of two particles once they have formed an encounter complex can be achieved by rotational diffusion. Rotational diffusion will have a significant effect if the value of a term, Y, is greater than about 0.2 (Shoup et al., 1981):

$$Y = a(D_R/D)^{0.5}$$

where a and D are defined above and D_R is the rotational diffusion constant of the vesicle. The value of Y can approach 0.3–0.6 for SUVs but is very small for LUVs. Consequently, rotational diffusion could enhance the effectiveness of SUVs to a small extent.

Fourth, Berg (1985) has estimated that weak binding of a protein to a membrane surface followed by two-dimensional diffusion could greatly enhance the search by the bound protein. Acidic phospholipids might therefore be more effective due to weak binding of C5b-7 through ionic attraction and such two-dimensional diffusion. Experimental demonstration of the influences of rotational and two-dimensional diffusion on this reaction, with separation from other explanations for the observed effects, would appear difficult.

Finally, differences between vesicles of different sizes and compositions could be explained by differences in the phospholipid surface related to insertion of C5b-7. Many studies have shown that SUVs have high surface curvature with accompanying strain and low interaction of the head groups on the outer phospholipid layer [e.g., Shuh et al. (1982) and Huang & Mason (1978)]. This may increase the frequency of occurrence of a structure needed for C5b-7 insertion such as exposure of the hydrocarbon region of the membrane. LUVs, with low surface curvature, show similar head group environments for inner- vs. outer-layer phospholipid head groups with less exposure of the hydrocarbon region of the bilayer. Consequently, insertion of C5b-7 into the SUV lipids may be more facile than into the LUV lipids. Since other factors considered above seem inadequate to explain the 20-fold selectivity for SUV lipids, surface curvature appeared the most attractive explanation for selectivity. The major energy barrier for C5b-7 insertion may be penetration through the polar head group region of the bilayer. This could also be the major explanation for selectivity for acidic phospholipids; the latter should show increased separation of head groups due to charge–charge repulsion.

The effectiveness of DMPC was only 0.20 times that of egg yolk PC. This selectivity was not due to the gel state since the same relative selectivity was observed at 33 °C. The explanation could be closely related to the ineffectiveness of LUVs; the short saturated hydrocarbon chains of DMPC should allow closer packing arrangements for the outer phospholipid layer, thereby decreasing the strain induced by high surface curvature in SUVs and decreasing exposure of the hydrocarbon side chains. In fact, it is possible that the limiting factor for C5b-7 insertion, in all cases, may be penetration through the lipid head group region of the bilayer.

Previous studies have shown much greater interaction of complement with cells containing an agent [A2C (Shin et al., 1981)] that increases membrane disorder. On the basis of our studies, it is possible that this agent functions by increasing exposure of the hydrocarbon regions of the membrane. However, membranes treated with this reagent also displayed the ability to bind C5b-6. An alternative explanation is that the negatively charged disordering agent functioned primarily by increasing the charge density of the membrane; both C5b-6 and C5b-7 binding are highly influenced by this parameter. In contrast, the fluid vs. gel state, a major change in phospholipid disorder, seemed to have little influence on C5b-7 binding.

Richards et al. (1986) found that complement released 100% of the vesicle contents of SUVs but only 25–40% of the contents of LUVs regardless of the amount of serum complement added. They attributed this to loss of the channel and failure to disperse all contents of LUVs. However, our results suggest an alternative explanation for these observations. Poor reaction of C5b-7 with LUVs, as shown here, would cause increased fluid-phase aggregation of C5b-7. Use of serum as the source of complement (Richards et al., 1986) provides that inhibitors (protein S; Podack et al., 1977) of membrane attack are added in increasing amounts along with complement. Depending on competitive reactions, it may be possible to reach an apparent saturation point where further increases in serum result exclusively in greater fluid-phase aggregation.

These studies suggest that the insertion of C5b-7 into membranes may consist of a relatively nonspecific hydrophobic interaction between protein segment and the hydrocarbon region of the membrane. Structures that enhance exposure of the hydrocarbon region or membranes that naturally have greater hydrocarbon exposure will be selected targets of C5b-7. Whether this is a relevant in vivo mechanism for producing attack selectivity for the MAC will require further studies.

Registry No. C5b-6, 84012-71-5; C7, 80295-57-4; C5b-7, 84012-72-6; DMPC, 13699-48-4; DPPC, 2644-64-6; DOPC, 4235-95-4.

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Reaction of Some Macrolide Antibiotics with the Ribosome. Labeling of the Binding Site Components†

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ABSTRACT: Radioactive carbomycin A, niddamycin, tylosin, and spiramycin, but not erythromycin, can be covalently bound to *Escherichia coli* ribosomes by incubation at 37 °C. The incorporation of radioactivity into the particles is inhibited by SH– and activated double bond containing compounds but not by amino groups, suggesting that the reactions may take place by addition to the double bond present in the reactive antibiotics. This thermic reaction must be different from the photoreaction described for some of these macrolides [Tejedor, F., & Ballesta, J. P. G. (1985) *Biochemistry 24*, 467–472] since tylosin, which is not photoincorporated, is thermically bound to ribosomes. Most of the radioactivity is incorporated into the ribosomal proteins. Two-dimensional gel electrophoresis of proteins labeled by carbomycin A, niddamycin, and tylosin indicates that about 40% of the radioactivity is bound to protein L27; the rest is distributed among several other proteins such as L8, L2, and S12, to differing extents depending on the drug used. These results indicate, in accordance with previous data, that protein L27 plays an important role in the macrolide binding site, confirming that these drugs bind near the peptidyl transferase center of the ribosome.

The macrolides form a rather large group of natural compounds, having in their chemical structure a macrocyclic ring closed by lactonization, and showing inhibitory activity against living organisms (Hamilton-Miller, 1973). There are a number of antibiotics of great clinical importance among them (such as erythromycin, spiramycin, etc.) which specifically inhibit bacterial protein synthesis (Vázquez, 1979).

The macrolides which inhibit protein synthesis bind at mutually exclusive sites on the ribosome (Fernández-Muñoz et al., 1971). On the basis of their mode of action, they can be classified into two main groups represented by erythromycin

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