Assembly of the Membrane Attack Complex of Complement on Small Unilamellar Phospholipid Vesicles†

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Received August 9, 1985

ABSTRACT: Light-scattering intensity was shown to be a reliable, direct, and quantitative technique for monitoring the assembly of the membrane attack complex of complement (proteins C5b-6, C7, C8, and C9) on small unilamellar phosphatidylcholine vesicles. The assembly on vesicles occurred in a simple fashion; complexes of C5b-7 bound noncooperatively to the vesicles, and final assembly of C5b-9 did not induce vesicle aggregation or fragmentation. When C5b-6 and C7 were mixed in the presence of vesicles but at molar protein/vesicle ratios of <1, there was quantitative binding of C5b-7 to the vesicles with no concomitant aggregation of C5b-7. If C7 was added at a slower rate, quantitative binding was obtained at molar C5b-7/vesicle ratios of up to 5. The latter observations (a) were consistent with the proposal that C5b-7 aggregation and membrane binding were competitive events and (b) defined conditions under which light-scattering intensity measurements could monitor C5b-9 assembly on vesicles without contribution from the fluid-phase assembly. The C8/C5b-7 ratio in the phospholipid–C5b-8 complex was 0.97 ± 0.12, and the maximum ratio of C9/C5b-8 in the final complex was 16.2 ± 2.0. One C9 molecule associated rapidly with each phospholipid–C5b-8, followed by slower incorporation of the remaining C9 molecules. The initial velocity of the slow phase of C9 addition was easily saturated with C9 and gave an activation energy of 37 kcal/mol. This was identical with the value measured for the analogous process in the fluid-phase assembly. The results indicated that the mechanism of C5b-8-dependent C9 polymerization on vesicles was indistinguishable from that of the fluid-phase assembly [Silversmith, R. E., & Nelsestuen, G. L. (1986) *Biochemistry* (preceding paper in this issue)]. Therefore, insertion of C9 into the membrane was not a part of the rate-limiting process.

Serum complement proteins C5b, C6, C7, C8, and C9 self-assemble on a phospholipid (PL)[1] membrane to form a cytolytic complex designated the membrane attack complex (MAC). The assembly is sequential and has been shown to occur in the manner (Muller-Eberhard, 1975):

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

C5b-6 is a stable complex and can be isolated from C7-deficient serum which has undergone complement activation (Podack et al., 1978). Combination with C7 produces the first complex that associates with lipid membranes. This trimeric complex can also associate with itself (Podack & Muller-Eberhard, 1978; Silversmith & Nelsestuen, 1986) or another plasma protein designated S protein (Podack et al., 1977; Podack & Muller-Eberhard, 1978; Dahlback & Podack, 1985).

Incorporation of C9 is essential for conferring maximal hemolytic activity to the complex; C5b-7 bound to erythrocytes causes no hemolysis while subsequent incorporation of C8 results in slow hemolysis which is greatly accelerated by C9. The complete C5b-9 complex is also necessary to form the doughnut-shaped lesions on the membranes of complement-lysed cells which have been visualized by electron microscopy (Podack et al., 1982; Bhakdi & Tranum-Jensen, 1984; Tschopp, 1984). The C9 protein alone can form polymers under denaturing conditions which display a tubule ultrastructure indistinguishable from the MAC lesion (Tschopp et al., 1982). The polymers are reported to have inserted into the phospholipid bilayer of synthetic vesicles if formed in their presence (Tschopp et al., 1982) just as C9 is purported to do in membrane-bound C5b-9 (Tschopp et al., 1982). The C9 polymer is resistant to dissociation by SDS, as is a large portion of the membrane-bound C5b-9 which consists mostly of C9 (Podack & Tschopp, 1982b; Podack, 1984; Tschopp et al., 1985). Therefore, it has been proposed that the tubule structure associated with MAC formation consists mostly of C9 and the role of C5b-8 is to catalyze formation of the C9 tubule (Yamamoto & Migita, 1982; Podack et al., 1982; Tschopp et al., 1985). Dankert et al. (1985), however, recently reported that tubule formation was not essential for expression of full lytic activity.

There is some disagreement as to the C9/C5b-8 stoichiometry in the final complex. Many laboratories have measured this stoichiometry using a variety of methods, and determinations range from 3 to 16 C9 molecules incorporated per C5b-8 [see Stewart et al. (1984) for a discussion]. Recently, Stewart et al. (1984) and Sims (1983) measured C9/C5b-8 ratios of (3–4)/1 by quantitation of radiolabeled C8 and C9 using erythrocytes or erythrocyte ghosts as the

† This work was supported by National Institutes of Health Grant HL 15728.
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‡ Supported by NIH Training Grant 5T32-GM07323 and the Louise T. Dosdall Fellowship, The University of Minnesota.

[1] Abbreviations: MAC, membrane attack complex; PL, phospholipid; fluid phase–C5b-7, –C5b-8, and –C5b-9, the MAC intermediates assembled in the absence of membrane as described in the preceding paper (Silversmith & Nelsestuen, 1986); phospholipid (or PL)–C5b-7, –C5b-8, and –C5b-9, the MAC intermediates bound to small unilamellar phospholipid vesicles; NBD-PE, *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine; Rho-DOPE, *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; *I*, intensity; PE, phosphatidylethanolamine; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-0852$01.50/0

target membrane. However, Tschopp estimated a C9/C5b-8 ratio of (12–18)/1 based on the independent observations that (a) the C9 polymer formed independent of C5b-8 consisted of 12–18 C9 monomers per tubule (Tschopp et al., 1984) and (b) one C5b-8 was associated with one tubule (Tschopp, 1984).

Investigation of the mechanism by which C5b-8 polymerizes C9 has been limited by the inability to directly observe the dynamics of MAC assembly over its entire time course. Sims & Wiedmer (1984) monitored C9 polymerization with a fluorescent derivative of C9. The fluorescence intensity of C9 labeled with fluorescein 5-isothiocyanate was quenched as the C9 polymerized in the presence or absence of C5b-8. With this technique, the activation energy for C9 polymerization by C5b-8 bound to synthetic vesicles (21–24 kcal/mol) and to sheep red blood cells ghosts (13 kcal/mol) was measured (Sims & Wiedmer, 1984). These values compared to a reported activation energy of 40 kcal/mol for the C5b-8-independent polymerization of C9 (Podack et al., 1982). The authors concluded that the role of C5b-8 may be to lower the activation energy of C9 polymerization. However, the fluorescence method is an indirect approach that is dependent on similar spacial relationships for each of the C9 molecules in the C9 polymer.

In the preceding paper (Silversmith & Nelsestuen, 1986), light-scattering intensity measurements were used to quantitatively monitor the assembly of the fluid-phase MAC. These studies showed that one C9 bound very rapidly to C5b-8 and the rate-limiting process involved incorporation of the remaining C9 molecules. The activation energy of this rate-limiting process (37.0 kcal/mol) was not significantly different from that for the spontaneous polymerization of C9 (40.8 kcal/mol).

The studies reported here show that light-scattering intensity can also be used to monitor the assembly of the MAC on small unilamellar phospholipid vesicles. This provides a rapid analytical technique that allows direct quantitative observation of the various stages of MAC assembly. It was found that assembly on phospholipid vesicles behaved in a simple manner; C5b-7 associated with the vesicles in a statistically random fashion, and the vesicles remained intact with no detectable aggregation or disintegration upon binding of the remaining MAC proteins. With this technique, the protein stoichiometry in the final complex was measured, and insight into the mechanism of C9 polymerization by phospholipid–C5b-8 was obtained.

Materials and Methods

Proteins. The preparation and characterization of complement components C5b-6, C7, C8, and C9 were described in the preceding paper (Silversmith & Nelsestuen, 1986). As in the preceding paper, the concentration of C5b-7 was based on the quantity of the limiting component (C5b-6 or C7) which was present and the reported molecular weights of C5b-6 (325 000) and C7 (120 000).

Vesicles. Small unilamellar vesicles were prepared by the method of ethanol injection (Kremer et al., 1977). For light-scattering intensity experiments, these vesicles consisted of highly purified phosphatidylcholine (PC; Sigma Chemical Co., St. Louis, MO). One milligram of PC was dried, dissolved in 0.5 mL of absolute ethanol, and injected in 5-μL aliquots into 10 mL of buffer (50 mM Tris/100 mM NaCl, pH 7.4) while stirring. The molecular weight of vesicles prepared by this method was measured by light-scattering intensity [eq 1b of Silversmith & Nelsestuen (1986)] and ranged from 4.0×10^6 to 6.0×10^6 in different preparations. Vesicles containing trace amounts of ^{14}C-phospholipid were prepared by injecting 300 μg of PC and 8 μg (1.2 μCi) of [^{14}C]DPPC (L-α-[1-^{14}C]dipalmitoylphosphatidylcholine; New England Nuclear, Boston, MA; 112 mCi/mmol) dissolved in 0.150 mL of ethanol into 4 mL of buffer. Vesicles prepared in this fashion had a specific activity of 4100 cpm/μg of phospholipid.

For fluorescence energy transfer experiments, vesicles containing 10% fluorescent-labeled phospholipid were prepared by injecting 900 μg of PC and 100 μg of either Rho-DOPE or NBD-PE (both from Avanti Polar Lipids Inc., Birmingham, AL) into 10 mL of Tris buffer.

Small unilamellar vesicles containing 5(6)-carboxyfluorescein in the interior were prepared by bath sonication followed by gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), as described in Silversmith and Nelsestuen (1986).

Vesicles were stored at 4 °C and were used within 3 days after preparation. Phospholipid concentration was determined by an organic phosphate assay (Chen et al., 1956) using a phospholipid/phosphorus weight ratio of 25.

Light-Scattering Intensity Measurements. Rayleigh light scattering at 90° was used to quantitate the binding of MAC proteins to small unilamellar phospholipid vesicles. The equations and their application to the fluid-phase assembly of the MAC were presented in Silversmith and Nelsestuen (1986). The general method used for monitoring protein binding to vesicles was described in Nelsestuen and Lim (1977). Excess light scattering which occurs upon binding of protein to vesicles is due to protein–vesicle interactions. The intensity from free protein is small or negligible compared to the intensity of vesicles or the protein–vesicle complex and can be subtracted when necessary. The excess light-scattering intensities of the vesicle solution before and after addition of protein can be used to find the molecular weight ratio of protein–vesicle complex/vesicle:

$$I_{s_2}/I_{s_1} = (M_{r_2}/M_{r_1})^2[(\partial n/\partial c)_2/(\partial n/\partial c)_1]^2 \qquad (1)$$

where I_s is the excess scattering intensity of the solute, M_r is the molecular weight, and $\partial n/\partial c$ is the refractive index increment. Subscript 1 refers to the vesicles and subscript 2 to the protein–vesicle complex. In these studies, this expression was used to quantitate the association of C5b-7, C5b-8, and C5b-9 with vesicles. The refractive index increment was assumed to be equal to the weight-averaged composition of each particle with $\partial n/\partial c = 0.19$ for pure protein and 0.17 for phospholipid (Nelsestuen & Lim, 1977). The ratios $M_{r(ves-C5b-7)}/M_{r(ves)}$, $M_{r(ves-C5b-8)}/M_{r(ves)}$, and $M_{r(ves-C5b-9)}/M_{r(ves)}$, computed by using eq 1, were used to calculate the molecular weight ratios of the various membrane-bound MAC intermediates:

$$[M_{r(ves-C5b-8)}/M_{r(ves)} - 1]/[M_{r(ves-C5b-7)}/M_{r(ves)} - 1] = M_{r(C5b-8)}/M_{r(C5b-7)} \qquad (2a)$$

$$[M_{r(ves-C5b-9)}/M_{r(ves)} - 1]/[M_{r(ves-C5b-8)}/M_{r(ves)} - 1] = M_{r(C5b-9)}/M_{r(C5b-8)} \qquad (2b)$$

The stoichiometries of proteins in the complexes were then calculated from these molecular weight ratios and the established molecular weights of the individual protein components (C5b-6, 325 000; C7, 120 000; C8, 150 000; C9, 71 000).

The $M_{r(protein-ves)}/M_{r(ves)}$ ratios obtained from eq 1 were also used to calculate the quantity of protein which bound to vesicles:

$$[M_{r(protein-ves)}/M_{r(ves)}]C_{ves} = C_{ves+bound\ protein} \qquad (3)$$

where c is the weight concentration (w/v). The weight concentration of vesicles was measured separately so the quantity

of bound protein was calculated.

The molecular weight of the vesicles was estimated from light-scattering intensity measurements and comparison to a standard as described in the previous paper [see eq 1b of Silversmith & Nelsestuen (1986)].

Light-scattering intensity measurements were made either with a Perkin-Elmer 44A fluorescence spectrophotometer at 320 nm or with the 488-nm laser instrument described by Pletcher et al. (1980). The standard buffer, 50 mM Tris/100 mM NaCl, pH 7.4, was used for the binding experiments and was filtered through a 0.22-μm filter immediately prior to use. Proteins were centrifuged for 5 min at 13000g in a Beckman microfuge just prior to experiments. Unless specified, all binding experiments were done at 25 °C. Temperature equilibration was achieved by a circulating ethylene glycol/water bath. The temperature of the sample was measured directly in the cuvette with a thermistor.

Fluorescence Energy Transfer. The method was similar to that described by Struck et al. (1981). A mixture of two populations of fluorescent vesicles (7.5 μg of 90% PC/10% Rho-DOPE and 2.5 μg of 90% PC/10% NBD-PE) in 1.5 mL of buffer was used. Fluorescence energy transfer from the NBD to the rhodamine moieties as a result of vesicle fusion, aggregation, or phospholipid exchange was estimated from the emission spectrum of the mixture between 500 and 620 nm with excitation of the NBD moiety at 452 nm.

Hydrodynamic Measurements. Quasi-elastic light scattering was used to determine the Z-averaged diffusion coefficients ($D_{20,w}$) of the protein–vesicle complexes. The theory, methods, and apparatus have been described (Pletcher et al., 1980). The Z-averaged hydrodynamic radius (R_h) was calculated from the diffusion coefficient by using the Stokes–Einstein relationship

$$R_h = kT/6\pi\eta D_{20,w}$$

where η is the viscosity of the solvent. For the measurements made on fractions from sucrose gradients, the $D_{20,w}$ and R_h values were calculated by using solvent refractive indexes and viscosities corresponding to the sucrose content of the fraction.

Sucrose Gradient Sedimentation. Sucrose solutions were prepared by using ultra-pure sucrose (Bethesda Research Labs, Bethesda, MD). Before use, the solutions were filtered with pressure through a Diaflo xm-10 ultrafiltration membrane (Amicon Corp., Danvers, MA) to remove particulate contaminants. After filtration, the sucrose solutions had a small excess scattering intensity which was proportional to the sucrose concentration and was subtracted from the measured light-scattering intensities of the samples. Linear sucrose gradients (11.6 mL, 5–20%) were poured on a 60% sucrose cushion (0.5 mL) in $^9/_{16}$ in. × 3.5 in. polyallomer tubes. Samples (0.5 mL) were applied to the top of the gradients by using a syringe, and tubes were centrifuged at 25 000 rpm and 20 °C with a Beckman SW41 rotor in a Beckman L2-65B ultracentrifuge. Fractions (0.8 mL) were collected by slowly pumping the gradient from the bottom of the tube. Tobacco mosaic virus (TMV, gift of Dr. Warren Gallagher) was run as a standard ($s_{20,w}$ = 187 S) in the sedimentation experiments. The $s_{20,w}$ value of the MAC–vesicle complex was estimated by direct comparison of its velocity, V, to that of TMV as described by Martin and Ames (1961):

$$s_{MAC\text{-}ves} = 187V_{MAC\text{-}ves}/V_{TMV}$$

Results

MAC Assembly on Phospholipid Vesicles Monitored by Light-Scattering Intensity. Figure 1 shows typical light-scattering intensity changes which occurred when C5b-6, C7, C8, and C9 were sequentially added to phospholipid vesicles (solid line). Also shown (dashed line) are the light-scattering intensity changes for identical quantities of MAC proteins added in the same manner but in the absence of vesicles. In the latter case, the initial intensity reading is that of the buffer and C5b-6 mixture adjusted to the same height as the phospholipid-containing mixture. The light-scattering intensity changes which occurred in the presence of vesicles were qualitatively similar but quantitatively quite different from those which occurred in the absence of vesicles.

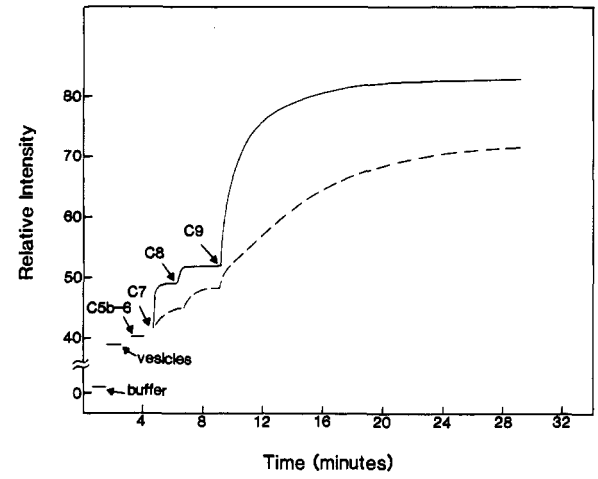

FIGURE 1: Light-scattering intensity changes when C5b-9 was assembled in the presence and absence of vesicles. The chart recorder was zeroed at the dark current of the instrument (emission shutter closed). C5b-6 (15 μL, 1.7 μg), C7 (7 μL, 3.5 μg), C8 (7 μL, 4.0 μg), and C9 (10 μL, 8.0 μg) were added at the designated arrows to 1.5 mL of buffer (dashed line) or to 1.5 mL of buffer containing 20 μg of PC vesicles (solid line). For the fluid-phase assembly (dashed line), the initial intensity was due to the C5b-6–buffer mixture and was adjusted in the figure to equal that for vesicles plus C5b-6. Both reactions then started at the same point on the chart (labeled C5b-6), and the subsequent relative changes were plotted. The lines drawn are a transposition of the chart tracings; the signal/noise ratio was ≥30/1 in all measurements. Light-scattering intensity is given in arbitrary units. Relative intensities within a given figure are directly comparable, but intensities in different figures are not comparable.

Addition of C5b-6 to vesicles gave a small intensity increase which was equal to that observed when C5b-6 was added to buffer alone. Consequently, there was no detectable binding interaction between C5b-6 and the vesicles. Preparations of C7, C8, and C9, however, caused negligible light-scattering intensity increases at the machine sensitivity used when added individually to either buffer or a vesicle solution. When C7 was added to C5b-6 and vesicles, an increase in intensity ($\Delta I_{C5b\text{-}7}$) was observed due to the formation of phospholipid–C5b-7. Addition of excess C8 resulted in a further light-scattering intensity increase (ΔI_{C8}) due to the association of C8 with phospholipid–C5b-7 to form phospholipid–C5b-8. Finally, addition of C9 caused a large time-dependent increase in light-scattering intensity which plateaued within about 10 min. Both the initial rates and the final intensities of these increases were significantly greater in the presence of vesicles than in their absence. All of the changes in light-scattering intensity were dependent on the presence of the proteins which bound earlier in the MAC assembly; for example, C8 did not show a light-scattering increase unless C5b-7 was present, and C9 did not cause changes unless C5b-8 was present.

The results showed a qualitative difference for MAC assembly in the presence vs. the absence of vesicles but did not establish that all of the MAC complexes were assembling on the membrane. It was imperative, however, to operate under

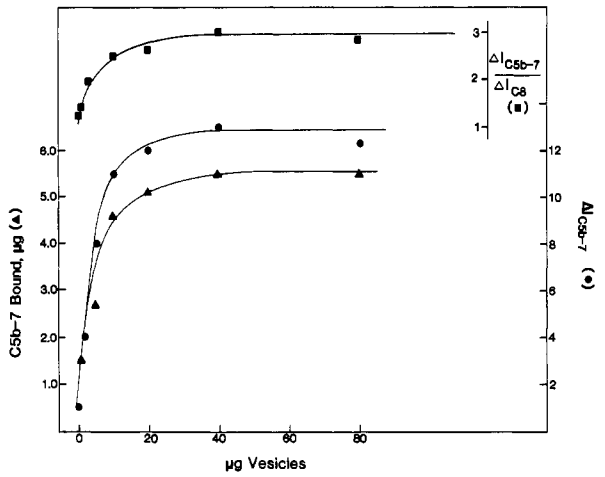

FIGURE 2: Addition of C5b-7 to varying quantities of phospholipid vesicles. C5b-6 and C7 (to make 5.5 μg of C5b-7) were added to PC vesicles (0–80 μg) in 1.5 mL of buffer. After the light-scattering signal stabilized, excess C8 (8.0 μg) was added. For each vesicle concentration, the light-scattering intensity change which occurred after addition of C7 [ΔI_{C5b-7} (●)] was measured. This value was compared to the intensity of the vesicles (I_{ves}) to determine the quantity of C5b-7 bound (▲) by using eq 3. The ratio of intensity changes which occurred upon addition of C7 and C8 ($\Delta I_{C5b-7}/\Delta I_{C8}$) is plotted at the top of the graph (■).

conditions where light-scattering contributions from the fluid-phase assembly were negligible so that observations could be attributed to MAC assembly on vesicles. Since aggregation of C5b-7 is competitive with its phospholipid association, higher concentrations of vesicles should increase the proportion of vesicle-bound C5b-7 until vesicle association is quantitative. The results indicated that this phenomenon did occur (Figure 2). Excess C5b-6 and a limiting quantity of C7 (to produce 5.5 μg of C5b-7) were added to varying quantities of vesicles. The absolute light-scattering intensity change upon addition of C7 (ΔI_{C5b-7}) increased to a maximum and then remained constant (Figure 2) as phospholipid concentration increased. The absolute light-scattering changes were used to calculate the quantity of bound C5b-7 (eq 3). This calculation was based on the initial assumption that the intensity increases were due entirely to protein–vesicle interaction without contribution from fluid phase–C5b-7. If this condition was met, the amount of bound protein should equal the amount of C5b-7 formed in the reaction (5.5 μg). If binding was not quantitative, however, the observed amount of bound protein would be an inaccurate determination and would be less than quantitative binding. Figure 2 demonstrates that quantitative binding was obtained at ≥40 μg of vesicles. Quantitative binding of this quantity of protein to 40 μg of vesicles corresponded to a C5b-7/vesicle ratio of 1.2. Below this quantity of vesicles, protein binding was less than quantitative, indicating that some fluid-phase aggregation occurred.

A ratio of light-scattering intensities ($\Delta I_{C5b-7}/\Delta I_{C8}$) was found to be a reliable indicator of the contribution to the light-scattering signal from the fluid-phase assembly. In the fluid-phase system, the ratio of these intensities was 1.27. This ratio was constant and corresponded to the association of 1.0 C8 per C5b-7. Figure 2 shows that the $\Delta I_{C5b-7}/\Delta I_{C8}$ ratio increased from 1.27, when no vesicles were present, to a saturating value of about 2.8, when all the C5b-7 was bound to the vesicles. The fact that a saturating value was obtained indicated that maximum binding of C5b-7 to the vesicles had been achieved.

Because C5b-7 self-aggregation and membrane binding are competitive and irreversible processes, decreasing the rate of formation of C5b-7 should decrease the concentration of C5b-7 present at any given time and thereby increase the likelihood of C5b-7 interaction with a vesicle vs. with another C5b-7 molecule. This was tested by decreasing the rate of addition of C7. At saturating quantities of vesicles (≥40 μg), slower addition of C7 had no effect on the result. However, at 10 μg of vesicles, four smaller additions of C7 resulted in essentially quantitative binding (95%) of C5b-7 to the vesicles compared to an apparent value of 77% when the C7 was added in one addition. Quantitative binding at these protein and lipid concentrations corresponded to 4.9 C5b-7 complexes per vesicle of M_r 4.0×10^6. Slow addition of C7 to 2.5 μg of vesicles resulted in an increase of apparent phospholipid–C5b-7 from 52% to 67%. Quantitative binding may not have been possible at this vesicle concentration since this would require each vesicle to accommodate 18 C5b-7 complexes. While it is possible that even slower formation of C5b-7 might increase the amount of vesicle-bound C5b-7 further, such attempts were not made.

The fact that the efficiency of binding was enhanced by slow addition of C7 was consistent with competition between self-aggregation and membrane association of C5b-7. It should be noted that the light-scattering results showed no indication of secondary processes such as vesicle aggregation or fusion which would cause large light-scattering intensity changes. The changes always remained at or below the theoretical values for protein binding.

Further Studies on Vesicle Fusion. Electron microscopy studies have indicated that C5b-9 induced fragmentation and aggregation of synthetic vesicles (Tschopp et al., 1982; Sims, 1984). Although the light-scattering data suggested that this did not occur, three further experiments independently confirmed that assembly of MAC proteins did not cause vesicle–vesicle association or fragmentation. In the first approach, vesicle fusion or aggregation was monitored by fluorescence energy transfer between rhodamine and NBD moieties, where each fluorescent probe was covalently incorporated into the bilayer of a separate vesicle population. Energy transfer could only occur if the two phospholipid populations were placed in close proximity to each other through aggregation or fusion. Figure 3 shows the fluorescence properties of the mixture of vesicles (7.5 μg of 10% Rho-DOPE/90% PC and 2.5 μg of 10% NBD-PE/90% PC). The major emission peaks of the NBD and rhodamine moieties are evident at 535 and 585 nm, respectively. The effects of vesicle aggregation are shown by addition of 10 mM $LaCl_3$ to this mixture (Figure 3). Fluorescence energy transfer from NBD to rhodamine is evident as the intensity of the NBD emission decreased by a factor of 2 and the rhodamine emission intensity increased by about 1.4-fold over that of the original mixture.

When C5b-6, C7, C8, and C9 were sequentially added to an identical mixture of fluorescent vesicles, there was no change in the fluorescence emission spectrum from that of the original mixture (Figure 3). This indicated that the MAC assembled on phospholipid vesicles without vesicle aggregation or fusion or exchange of phospholipid molecules between vesicles. Light-scattering intensity measurements showed that the proteins bound to the labeled vesicles.

A second experiment examined the sedimentation characteristics of the MAC–vesicle complex in sucrose density gradients. The MAC was assembled on PC vesicles (14 μg) which contained [^{14}C]DPPC (4100 cpm/μg of phospholipid). The quantity of added protein corresponded to eight C5b-7 complexes per vesicle of M_r 4.0×10^5. Under these conditions, some fluid-phase assembly would occur.

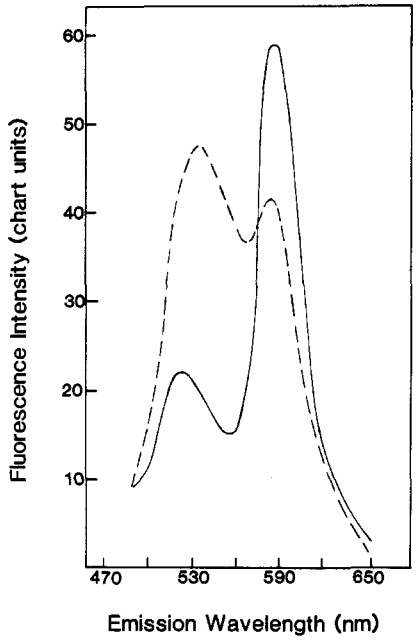

FIGURE 3: Energy transfer between two fluorescent-labeled vesicle populations as a measure of vesicle fusion, aggregation, or phospholipid exchange. The emission spectra of a mixture of two populations of vesicles (7.5 μg of 10% Rho-DOPE/90% PC and 2.5 μg of 10% NBD-PE/90% PC in 1.5 mL of buffer) alone (dashed line) and in the presence of 10 mM $LaCl_3$ (solid line) are shown. The emission spectrum in the presence of 10 μg of C5b-9 exactly superimposed that of the mixture alone (dashed line). The spectra were recorded on the Perkin-Elmer 44A spectrofluorometer (chart speed, 5 mm/min; scan speed, 30 nm/cm). The signal/noise ratio was >50/1.

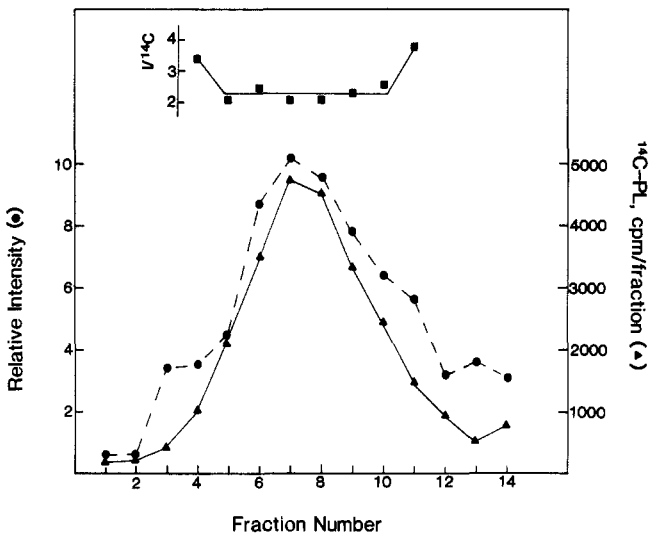

FIGURE 4: Sedimentation of phospholipid–C5b-9 in 5–20% sucrose density gradients. C5b-9 was assembled on small unilamellar PC vesicles (14 μg) which were trace-labeled with [^{14}C]DPPC (4100 cpm/μg of PL). Centrifugation was for 3 h at 25 000 rpm and 20 °C in a Beckman SW41 rotor. A portion (0.1 mL) of each fraction (total volume was 0.8 mL) was assayed for ^{14}C cpm (▲), the remaining 0.7 mL was centrifuged (5000 rpm at 20 °C), and the light-scattering intensity (●) was measured by using the laser light-scattering instrument. The light-scattering intensities were corrected for background scattering of the sucrose solution which was <25% of the total light scattering. The relative intensity/^{14}C ratio ($I/^{14}$C) (■) was calculated for each fraction.

As evident by the sedimentation profile (Figure 4), the ^{14}C-labeled vesicles sedimented as a single symmetric peak around a midpoint $s_{20,w}$ value of 62 *S*. The ratio of ^{14}C to light-scattering intensity was approximately constant across the peak, implying that the phospholipid-containing fractions

Table I: Hydrodynamic Characteristics of MAC–Vesicle Complexes Measured by Quasi-Elastic Light Scattering[a]

particle	R_h (Å)	$D_{20,w}$ (cm^2/s × 10^8)
phospholipid vesicles	211 ± 1	10.1 ± 0.1
phospholipid–C5b-7	232 ± 2	9.2 ± 0.1
phospholipid–C5b-8	242 ± 5	8.8 ± 0.2
phospholipid–C5b-9	275 ± 5	7.8 ± 0.2

[a] Phospholipid–C5b-9 was assembled by sequential addition of C5b-6 (2.2 μg), C7 (2.4 μg), C8 (5 μg), and C9 (8 μg) to PC vesicles (10 μg). Hydrodynamic measurements were made after each addition. Scattering from free protein had a negligible effect on the results. The final MAC/vesicle ratio was 3.9 as determined by light-scattering intensity measurements. Sample volume was 0.8 mL. The error represents the variation in repetitive measurements on the same sample.

consisted of complexes of essentially homogeneous molecular weight. This result confirmed that vesicle aggregation was negligible and that C5b-9 complexes were distributed approximately evenly over the population of vesicles. Even distribution would occur if C5b-7 bound randomly to the vesicles, a necessary property for quantitative interpretation of light-scattering intensity changes in a protein–vesicle system. The intensity/^{14}C ratio increase at the upper edge (higher fraction numbers) of the sedimentation peak was due to the shoulder on the side of the light-scattering intensity peak (fractions 10–12). This shoulder was most likely due to fluid phase–C5b-9 which sedimented in those fractions ($s_{20,w}$ = 33–60 S; Silversmith & Nelsestuen, 1986). The $D_{20,w}$ values of the particles in fractions 7 and 8 were 7.1 × 10^{-8} and 7.4 × 10^{-8} cm^2/s, corresponding to R_h values of 300 and 285 Å, respectively.

The measured $s_{20,w}$ value, coupled with the *Z*-averaged $D_{20,w}$ and a calculated partial specific volume of the particle (0.84 cm^3/g), indicated that the protein–vesicle complex had a molecular weight of 1.1 × 10^7. Given a molecular weight of 1.7 × 10^6 for the MAC complex (see below), each vesicle (M_r 4.0 × 10^6) had an average of 4.5 MAC complexes bound. Therefore, a sedimentation coefficient of 62 S was consistent with the expected value for the assembly of the MAC on monomeric vesicles. The width of the peak may reflect the random distribution of complexes on the vesicles (see below) and/or the polydispersity of the original vesicles. All the ^{14}C in the profile was associated with the 62 S peak, further indication that there was no vesicle fragmentation or very large particles due to vesicle aggregation. The partial specific volume of the protein–vesicle complex was a weighted average of that of vesicles (1.0 cm^3/g) and protein (0.72 cm^3/g). The protein/phospholipid ratio used in this calculation was estimated by light-scattering measurements.

Finally, hydrodynamic data showed that only small changes in the *Z*-averaged R_h occurred when the MAC was assembled on vesicles. When C5b-9 was assembled on the vesicles with a MAC/vesicle ratio of 4/1, the R_h increased from 211 to 275 Å (Table I). If the vesicles had aggregated, very large changes would be expected to occur: vesicle aggregation would result in domination of the *Z*-averaged radius by the largest particles. The R_h (275 Å) for phospholipid–C5b-9 was very similar to the R_h (285–300 Å) of the complex which had been separated from free protein by centrifugation (see above). This confirmed that the presence of free protein did not affect the *Z*-averaged hydrodynamic values or the weight-averaged light-scattering intensity measurements.

Experiments with vesicles containing 5(6)-carboxyfluorescein confirmed that C5b-7 associated with vesicles in a random fashion. The titration of these vesicles with C7 was described in the preceding paper [Figure 3 of Silversmith & Nelsestuen (1986)] and used there to demonstrate the full

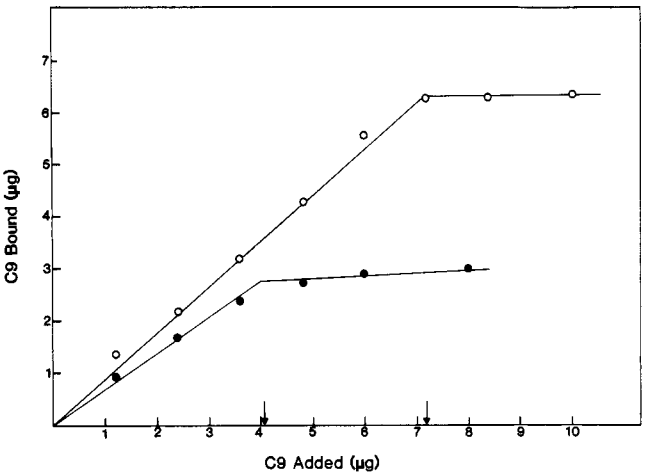

FIGURE 5: Incorporation of C9 into phospholipid- and fluid phase–C5b-8. Aliquots of C9 were added to phospholipid–C5b-8 [3.4 μg of C5b-8 bound to 40 μg of phospholipid (○)] or fluid phase–C5b-8 [3.4 μg, (●)] in 1.5 mL of buffer. The quantity of C9 bound after each solution was calculated from the resultant light-scattering intensity increases [eq 3 for the phospholipid system; see Silversmith & Nelsestuen (1986) for calculation in the fluid-phase system]. The arrows on the abscissa indicate the quantity of C9 necessary to saturate fluid phase–C5b-8 (left) and phospholipid–C5b-8 (right).

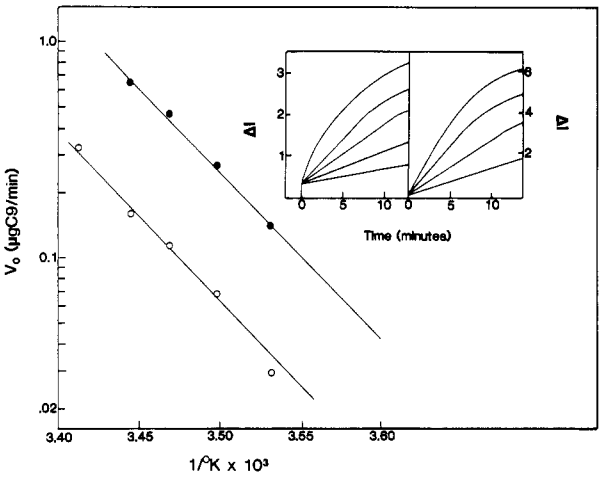

FIGURE 6: Arrhenius plot of C9 incorporation into phospholipid–C5b-8 and fluid phase–C5b-8. C9 (8.0 μg) was added to C5b-8 (3.3 μg) which was formed in the absence of vesicles (○) or in the presence of 20 μg of PC vesicles (●) in 1.5 mL of buffer. C5b-8 was assembled at room temperature and then equilibrated at the appropriate temperature before C9 addition. Initial velocities were measured at temperatures ranging from 10 to 20 °C. Inset: Time courses for initial velocity data; C9 was added at zero time, and the relative increase in intensity over that of fluid phase- or phospholipid–C5b-8 is shown. Left inset: experiment in the absence of vesicles at temperatures, from bottom to top, of 10.0, 13.0, 15.3, 17.2, and 20.2 °C. Right inset: experiment in the presence of vesicles at temperatures of, from bottom to top, 10.0, 13.0, 15.3, and 17.2 °C. The initial velocity at 20 °C was too fast for an accurate measurement in the presence of vesicles. Note that the intensity scale for the (+) vesicle data (right) is half of that for the (−) vesicle data (left).

functionality of C7 preparations. The data points relating moles of C7 added to moles of vesicles lysed coincided with a theoretical Poisson distribution, implying that C5b-7 added randomly to vesicles and was unaffected by the presence of other C5b-7 complexes bound to a vesicle.

It therefore appeared that the vesicle-containing system behaved in a simple fashion. C5b-7 bound randomly to the vesicles, followed by incorporation of C8 and C9. The assembly caused neither vesicle aggregation or vesicle disintegration. Therefore, under conditions where there was no fluid-phase assembly, light-scattering intensity changes could be used to quantitatively monitor the assembly of the MAC on phospholipid vesicles.

Interpretation of Light-Scattering Data and C8/C5b-7 Stoichiometry. At C5b-7/vesicle ratios where there was quantitative binding of C5b-7 to vesicles, $\Delta I_{C5b\text{-}7}$, $I_{C5b\text{-}8}$, and I_{ves} were used to calculate the number of C8 molecules bound to each C5b-7 (eq 2a). This stoichiometry was 0.97 ± 0.12 (1 S.D.) for all the titrations done under these conditions. This calculation was dependent on, and therefore strongly indicated, the absence of vesicle aggregation or fragmentation and accurate molecular weight ratios of C5b-7 and C8. This observed stoichiometry agreed with previous determinations of one C8 per C5b-7 in the membrane-bound MAC.

C9 Incorporation into Phospholipid–C5b-8. Small portions of C9 were added to phospholipid–C5b-8. Each addition resulted in a small increase in scattering intensity until saturation with C9 was achieved. The intensity at which the signal saturated was indistinguishable form the final intensity observed when excess C9 was added in one aliquot to the same quantity of phospholipid–C5b-8 (as in Figure 1). A parallel experiment was done in the absence of vesicles. The quantity of C9 bound after each addition was determined for both titrations (in the presence and absence of membranes) by using eq 3. There was essentially quantitative binding of C9 to both phospholipid–C5b-8 and fluid phase–C5b-8 until saturation of C9 binding sites was reached (Figure 5). These results indicated that C9 had a very high affinity for both fluid phase–C5b-8 and phospholipid–C5b-8.

Two independent properties of the experiment in Figure 5 indicated that phospholipid–C5b-8 could accommodate about twice as many C9 molecules as the fluid phase–C5b-8. The quantity of C9 required to saturate the light-scattering intensity change for fluid phase–C5b-8 was about half of that required to saturate an identical quantity of phospholipid–C5b-8 (see arrows on abscissa, Figure 5). Furthermore, at saturating C9, the final quantity of C9 bound to fluid phase–C5b-8 was about half of that bound to phospholipid–C5b-8 (see ordinate, Figure 5). The C9/C5b-8 ratio at saturating C9 (Figure 5) was 6.8 for fluid phase–C5b-8 and 15.6 for phospholipid–C5b-8. The value for fluid phase–C5b-8 obtained here correlated well with repetitive measurements of this stoichiometry reported in the preceding paper (Silversmith & Nelsestuen, 1986).

The C9/C5b-8 ratio of phospholipid–C5b-9 was also determined by calculation of the molecular weight ratio $M_{r(C5b\text{-}9)}/M_{r(C5b\text{-}8)}$ when C9 was saturating (eq 1 and 2b). This calculation was based solely on the ratio of intensity changes induced by C5b-8 and C5b-9 and did not require knowledge of the weight concentration of phospholipid or protein. The final intensity of phospholipid–C5b-9 in this calculation was independent of the manner of addition of C9 (in small aliquots or one large addition of excess C9). Using this approach, the C9/C5b-8 stoichiometry was determined to be 16.2 ± 2.0 (n = 9, 1 S.D.). The C9/C5b-8 stoichiometry did not vary significantly at C5b-9/vesicle ratios between 0.6 and 3.0. The stoichiometry of the MAC bound to small unilamellar vesicles therefore appeared to be $C5b_1C6_1C7_1C8_1C9_{14\text{-}18}$, implying that the molecular weight of the protein complex, C5b-9, which is bound to vesicles was about 1.7×10^6.

Kinetics of C9 Incorporation. The time course of C9 incorporation into phospholipid–C5b-8 was characterized by a small rapid intensity increase followed by a slow linear increase which finally plateaued (Figure 6, inset). The initial rapid increase was very small but detectable and reproducible. The error in measurement of the rapid increase was relatively large due to its small intensity compared to that of phospholipid–

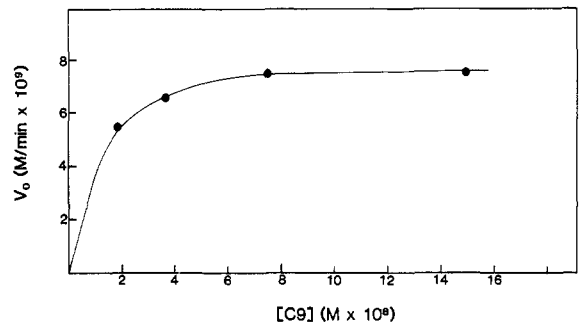

FIGURE 7: Initial velocity of the association of C9 with phospholipid–C5b-8 as a function of C9 concentration. C9 [2–16 μg; (2.8–22.5) $\times$ 10^{-11} mol] was added to phospholipid–C5b-8 (3.3 μg; 5.5 $\times$ 10^{-12} mol of C5b-8 bound to 20 μg of vesicles) in 1.5 mL of buffer which was equilibrated at 14.8 °C. The slope of the light-scattering intensity increase was used to calculate the initial velocity (eq 3).

C5b-8 ($\leq$5%). Precise measurement was therefore difficult despite the very large signal to noise ratio obtained in these studies. The magnitude of the rapid increase corresponded to 0.75 $\pm$ 0.25 C9 molecule per protein complex of phospholipid–C5b-8 (eq 2b). This value did not vary at MAC/protein ratios between 1 and 3. Like the fluid phase–C5b-8, phospholipid–C5b-8 bound one C9 rapidly, followed by the slower incorporation of the remaining C9 molecules.

The initial velocity of subsequent C9 incorporation into phospholipid–C5b-8 was determined by measuring the initial slope of the slow phase of the light-scattering intensity change. For the quantities of proteins used (2–6 μg of C5b-7), this initial slope was easily measurable at temperatures between 10 and 17.5 °C. The slope was converted to micrograms of C9 incorporated per minute (eq 3). Figure 7 shows the initial velocities measured when increasing amounts of C9 were added to phospholipid–C5b-7 at 14.8 °C. The results indicated that this initial velocity was dependent on C9 concentration up to a C9 concentration corresponding to about four C9 per C5b-8. Therefore, the rate-limiting step of C9 incorporation into phospholipid–C5b-8 occurred after the initial association of C9 with each C5b-8 and before the addition of the next C9. This is similar to the fluid-phase system where the initial velocity was sensitive to C9 concentration up to about two C9 per C5b-8 (Silversmith & Nelsestuen, 1986).

The initial velocity of C9 incorporation was measured at various temperatures to determine the activation energy of C9 incorporation into phospholipid–C5b-8. A parallel experiment was performed in an identical manner except in the absence of vesicles. In both experiments (in the presence and absence of vesicles), the initial velocity was derived from the slope of the time-dependent intensity increase which occurred after the rapid intensity increase (Figure 6, inset). The Arrhenius plots (Figure 6) showed that the activation energies in the presence and absence of vesicles were indistinguishable and corresponded to 37 kcal/mol. The rate of C9 incorporation into C5b-8 (micrograms of C9 per minute), however, was consistently about 4 times greater in the presence of vesicles.

DISCUSSION

Ninety degree Rayleigh light scattering has been used in the past as a direct and quantitative method to measure protein binding to small unilamellar vesicles [e.g., see Nelsestuen & Lim (1977) and Pusey et al. (1982)]. Because the light-scattering intensity from free protein is often negligible compared to the intensity of the vesicles or protein–vesicle complexes, corrections for light scattering by free protein are small or negligible. Protein binding to the vesicles can be quantitated by measuring a ratio of intensities: the intensity of the vesicles alone to the intensity of the vesicles with bound protein. The results demonstrated that this method was applicable to the assembly of the membrane attack complex on vesicles using purified components C5b-6, C7, C8, and C9. Application of this technique provided a direct and quantitative method for measurement of stoichiometries of the proteins in the complex and the kinetics of associations. Analysis did not require derivatization of the proteins or manipulation of products.

It was necessary to show that the changes which occurred when the MAC proteins were added to vesicles were due only to the association of the proteins with the vesicles. Several approaches showed that C5b-9 did not induce vesicle fusion, aggregation, or phospholipid exchange, all of which would affect interpretation of the light-scattering results. One experiment showed that C5b-9 did not induce detectable energy transfer between fluorophores in two populations of vesicles. This technique would be sensitive to small amounts of vesicle fusion, aggregation, or phospholipid exchange; vesicle aggregation of the mixture induced by $LaCl_3$ resulted in a high efficiency of energy transfer. A second experiment showed that phospholipid–C5b-9 complexes sedimented in a symmetric peak with an $s_{20,w}$ value of 62 S. This value correlated closely with the anticipated value for particles which would result from the association of the added protein with monomeric vesicles. The light-scattering intensity/^{14}C-phospholipid ratio was constant across the peak, indicating a relatively homogeneous population of particles. The sedimentation results also showed no low or very high molecular weight phospholipid components. In another approach, the hydrodynamic radius of the vesicle–protein particle underwent small increases upon assembly of the MAC on vesicles (4 MAC/vesicle). Since these values were derived from Z-averaged diffusion constants, large changes would be expected if vesicle aggregation had occurred.

All evidence, therefore, indicated that the vesicles remained intact and monomeric during MAC assembly. This conclusion contrasted with that of Sims (1984) and Tschopp et al. (1982), who reported C5b-9-dependent aggregation and fragmentation of unilamellar PC vesicles of large (500–10000 Å) and small (210 Å) diameters, respectively. These latter observations were made by electron microscopy, and preparation and manipulation of the sample may have affected the results. There have been reports of C5b-9-dependent release of phospholipids from *Escherichia coli* (Inoue et al., 1977) and liposomes made from erythrocyte and *E. coli* membrane constituents (Kinoshita et al., 1977). Shin et al. (1977) reported C5b-9-dependent release of phospholipid from synthetic PC liposomes. However, in these experiments, the liposomes were prepared with Forsmann antigen incorporated into the bilayers, and complement was activated by addition of anti-Forsmann antigen and large quantities of guinea pig serum. Our results showed that in a purified system no vesicle fragmentation occurred.

The light-scattering intensity changes which occurred during fluid-phase assembly of the MAC were qualitatively similar to those of the vesicle-bound assembly. Because the fluid-phase particles were similar in mass to the phospholipid vesicles, the fluid-phase assembly would interfere with interpretation of light-scattering data. However, at sufficiently low C5b-7/PL ratios ($\sim$1 C5b-7/vesicle), negligible fluid phase–C5b-7 was formed. If C7 was added slowly so that the C5b-7 concentration was maintained at a low level, the C5b-7/vesicle ratio could be increased to $\sim$5 C5b-7/vesicle with negligible concomitant fluid-phase–C5b-7 formation. This result was consistent with competition between C5b-7 aggregation and

membrane binding as reported by Podack et al. (1978a).

The ratio of C8/C5b-7 in phospholipid–C5b-8 was 1/1. This ratio was determined by using ratios of light-scattering intensities and was dependent on accurate molecular weights for C5b-6, C7, and C8. This 1/1 ratio was in agreement with previous reports for the vesicle-bound complex, indicating that the light-scattering changes reflected only protein–vesicle associations. A C8/C5b-7 ratio of 1.0 has also been measured in the fluid-phase assembly (Silversmith & Nelsestuen, 1986), confirming that there is one C8 binding site on C5b-7 which is independent of the presence of phospholipid.

The C9/C5b-8 ratio under conditions of saturating C9 was (16 ± 2)/1. Tschopp (1984) reported a similar ratio based on electron microscopic studies on both vesicles and erythrocytes which showed that there was one C5b-8 associated with each MAC tubule. Each tubule consisted of 12–18 C9 molecules (Tschopp et al., 1984), implying a C9/C5b-8 ratio of (12–18)/1. However, a C9/C5b-8 ratio of only (3–4)/1 has been reported by Stewart (1984) and Sims (1983). Both these studies involved quantitation of radiolabeled proteins and used erythrocytes or resealed erythrocyte ghosts as the target membrane. Other C9/C5b-8 ratios have been obtained from densitometric scans of electrophoretic gels of C5b-9 extracted from membrane by detergents [e.g., see Ware & Kolb (1981) and Bhakdi & Tranum-Jensen (1983)] and by measuring the molecular weight of the detergent-extracted MAC by sucrose gradient sedimentation (Biesecker et al., 1979; Bhakdi et al., 1981; Ware et al., 1981).

To reconcile the reported C9/C5b-8 ratio measured on erythrocytes [(3–4)/1] with the C9 tubule, it has been proposed that C5b-8 complexes may cluster on the membrane and each cluster is associated with one tubule. This would give a lower average C9/C5b-8 ratio. This proposal was supported by Podack et al., (1982) on the basis of their observation that the C9/C5b-8 ratio decreased (from 16 to 6) as the C5b-8/erythrocyte ratio increased (from 2500 to 25 000). Stewart et al. (1983), however, reported that the C9/C5b-8 ratio of (3–4)/1 was independent of the C5b-8/erythrocyte ratios from 25 to 4000 and concluded that clustering of C5b-8 complexes probably did not occur. Tschopp (1984), in direct electron microscopy studies, reported that only one C5b-8 was associated with a lesion on both sheep erythrocytes and vesicles. The measurements on vesicles, however, used C5b-7/vesicle ratios of <1, and clustering may only occur after two or more C5b-7 complexes have bound to the same vesicle. Our studies indicated that the C9/C5b-8 ratio did not vary significantly at C5b-7/vesicle ratios between 0.6 and 3. This implied that clustering did not occur under these conditions or that clustering did not affect the C9/C5b-8 ratio.

The apparent contradiction between the lower stoichiometries and the tubule of $(C9)_{12-18}$ could also be explained if only a portion of erythrocyte–C5b-8 could accommodate the number of C9 needed to form a full tubule. Bhakdi & Tranum-Jensen (1984) and Tschopp (1984) observed that the heterogeneity in the ultrastructure of MAC lesions on erythrocytes was due to varying C9/C5b-8 ratios. Even in the presence of saturating C9, many incomplete tubules were formed on erythrocyte membranes (Bhakdi & Tranum-Jensen, 1984). Therefore, some of the C5b-8 complexes may be associated with small numbers of C9(0–2, perhaps) and may not even be visible by electron microscopy under the conditions employed. This would result in an overall average of less than the maximal 12–18 C9/C5b-8. The inability of a portion of the C5b-8 complexes to incorporate the full C9 tubule may be due to steric obstruction or other complexities of the erythrocyte membrane. Evidence presented in the preceding paper (Silversmith & Nelsestuen, 1986) suggested that about half of the C5b-8 complexes formed in the fluid phase were incompetent of C9 addition. C5b-8 bound to vesicles in a purified system may have no obstructions and accommodate the full tubule, as indicated by the results obtained here.

Kinetic studies showed that incorporation of C9 into phospholipid–C5b-8 had characteristics similar to that of the fluid-phase assembly. In both systems, there was a rapid incorporation of one C9 per competent C5b-8, followed by slow incorporation of the remaining C9 molecules. The initial slow velocity of C9 incorporation was saturated with respect to C9 at low C9/C5b-8 ratios. The rate of C9 incorporation was about 4-fold faster in the presence of vesicles. However, the activation energies of C9 incorporation appeared indistinguishable (37 kcal/mol) in the presence and absence of vesicles. These results supported the hypothesis that, while only about half of the C5b-8 in fluid phase–C5b-8 appear to be functional, *functional* fluid-phase–C5b-8 complexes behaved in essentially the same manner as phospholipid–C5b-8.

The identity of the activation energies of C5b-8-dependent C9 polymerization in the presence and absence of vesicles implied that phospholipid was not involved in the rate-determining step. This conclusion was consistent with the mechanistic schemes presented in the preceding paper (Silversmith & Nelsestuen, 1986) where the rate-limiting step of C5b-8-dependent C9 polymerization was a conformational change of C9 to an activated state, C9′, which could occur in the absence of C5b-8. The result was also in agreement with Sims & Wiedmer (1984), who observed that the activation energy of C9 incorporation was not affected by the rigidity of the membrane bilayer.

The absolute value of the activation energy obtained here (37 kcal/mol) was significantly greater than that measured by Sims on phospholipid vesicles (21–24 kcal/mol). The latter measurement used energy transfer between fluorescent-labeled C9 and was dependent on similar spacial relationships between all C9 molecules in C5b-9. In a comparison of this activation energy to that of spontaneous C9 polymerization, Sims concluded that the role of C5b-8 may be to lower the activation energy of C9 polymerization. However, our data suggested that the role of C5b-8 may *not* include lowering of the activation energy of C9 polymerization. Possible alternative roles of C5b-8 were outlined in the preceding paper (Silversmith & Nelsestuen, 1986).

The observations obtained in this study indicate that the mechanisms by which phospholipid–C5b-8 and fluid phase–C5b-8 catalyze the polymerization of C9 are very similar. Therefore, the schemes presented in Silversmith and Nelsestuen (1986) which describe possible mechanisms of C5b-8-dependent C9 polymerization should also be applicable to the vesicle-bound system. The results did not show how and when the presence of phospholipid fits into the C9 assembly process. They did imply that the insertion of C9 into the membrane, when or if it occurs, must be facile and non rate limiting. The ability to use light scattering in a direct quantitative analysis of MAC assembly should allow many further studies on the physicochemical nature of this assembly.

ACKNOWLEDGMENTS

We thank Dr. Victor Bloomfield for use of the apparatus for quasi-elastic light scattering.

Registry No. Complement C5b, 80295-55-2; complement C6, 80295-56-3; complement C5b-6, 84012-71-5; complement C7, 80295-57-4; complement C5b-7, 84012-72-6; complement C8,

80295-58-5; complement C5b-8, 82903-91-1; complement C9, 80295-59-6; complement C5b-9, 82986-89-8.

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Production and Characterization of Monoclonal Antibodies against the Two Subunits Proteins B1 and B2 of *Escherichia coli* Ribonucleotide Reductase†

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Received June 26, 1985

ABSTRACT: Ribonucleotide reductase from *Escherichia coli* consists of two nonidentical subunits, named protein B1 (170 000) and protein B2 (87 000). We purified and characterized five monoclonal antibodies against B1 and three against B2 from hybridomas obtained by fusion of spleen cells from immunized mice and the myeloma cell line P3-X63Ag8. All are of the IgG_1 class with a high affinity for the antigen with dissociation constants in the nanomolar range. Four of the anti-B1 monoclonals and all three anti-B2 monoclonals neutralize reductase activity while one anti-B1 monoclonal binds tightly to B1 without affecting its activity. Fab fragments prepared from three anti-B1 monoclonals had similar dissociation constants. The anti-B1 monoclonals interacted with separate epitopes while two of the anti-B2 monoclonals appeared to react with the same epitope. In the case of B1, various allosteric states of the protein induced by binding of effectors had no apparent effect on the interaction with monoclonals, nor did their binding prevent subsequent binding of effectors. With B2, binding of monoclonals did not affect the typical electron paramagnetic resonance spectrum of the protein and thus did not involve either the tyrosyl free radical or the iron center of B2. All neutralizing antibodies interfered with the interaction between the two subunits, explaining their effect on enzyme activity, since active ribonucleotide reductase consists of a B1–B2 complex.

Ribonucleotide reductase from *Escherichia coli* catalyzes the reduction of the four common ribonucleoside diphosphates to the corresponding deoxyribonucleotides and provides the cell with the proper supply of precursors for DNA (Thelander & Reichard, 1979; Lammers & Follmann, 1983). The enzyme has been obtained in pure form, its complete nucleotide sequence was recently published (Carlsson et al., 1984), and work is in progress to determine its 3D structure by X-ray crystallography (Joelsson et al., 1984). The protein is made

† This work was supported by grants from the Swedish Medical Research Council, Magn. Bervall's Foundation, and the Wallenberg Foundation.

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0006-2960/86/0425-0860$01.50/0