Ninth Component of Complement: Self-Aggregation and Interaction with Lipids†

John R. Dankert,‡ John W. Shiver,§ and Alfred F. Esser*,‡
Laboratory for Structural Biology, Department of Comparative and Experimental Pathology, and Department of Chemistry, University of Florida, Gainesville, Florida 32610

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ABSTRACT: We have investigated environmental conditions that might be of importance for the polymerization of the ninth component (C9) of human complement. In disagreement with earlier reports summarized by Tschopp et al. [Tschopp, J., Müller-Eberhard, H. J., & Podack, E. R. (1982) *Nature (London) 298*, 534–538] we find no evidence for significant aggregation or loss of hemolytic activity of C9 when incubated at 37 °C even after 12 days of incubation. Higher temperatures cause denaturation of the protein and formation of stringlike aggregates. In contrast, short-term proteolysis with 1% (w/w) trypsin at room temperature causes rapid polymerization of part of the C9 into tubular structures (poly-C9), and the remainder of the monomeric C9 is digested. This polymerization reaction is inhibitable by trypsin inhibitor; α-thrombin and proteinase K are ineffective in creating polymers. A second discrepancy to the earlier reports is our finding that monomeric C9 immediately interacts with small unilamellar lipid vesicles (SUV) without a required heating step. As a result of this interaction about half of the C9 aggregates to form strings and tubules, and these aggregates cause agglutination of vesicles. The other half of the C9 associates with a second population of SUV without causing a change in Stokes' radius of these vesicles, and no proteinaceous structures are detectable on the vesicle surface by electron microscopy. When these two vesicle populations are tested for their membrane integrity, no release of an encapsulated fluorescent marker can be detected, nor is there leakage of potassium ions across the bilayer membrane since a membrane diffusion potential can be developed. Thus, we cannot confirm the earlier postulate that C9 is a cytotoxic molecule but conclude that neither monomeric nor polymerized C9 by itself is membranolytic in general. Monomeric C9 interacts with SUV above or below the lipid phase transition, but it does not associate with large multilamellar vesicles, indicating that the lipid packing density may be of importance for protein penetration. The relevance of this polymerization reaction to the function of C9 is uncertain. It is conceivable that the intermediate C5b-8 complex causes C9 aggregation in situ either because of an intrinsic proteolytic activity or because it changes the lipid packing in its vicinity, thereby allowing C9 to penetrate into the bilayer and subsequently to aggregate. We also consider that the polymerization process is a side reaction that functions to deactivate "active" C9 molecules.

The ninth component of complement (C9)[1] is a plasma glycoprotein that acts as the final component during the assembly of either the lytic membrane attack complex, MC5b-9, or its nonlytic counterpart, the fluid-phase SC5b-9 complex (Müller-Eberhard et al., 1968; Müller-Eberhard, 1980; Mayer, 1972; Esser, 1982). The functional activity of this protein was first described by Linscott & Nishioka (1963) in a fraction from guinea pig serum which they called C'3d, and the mode of action of this fraction was outlined by Nelson et al. (1966). The isolation and purification of human C9 were first achieved by Hadding & Müller-Eberhard (1969), and the porcine protein was characterized by Götze et al. (1967, 1968). These early reports indicated that the protein was sensitive to heating and also could be inactivated by metal ions.

Inactivation of C9 by heating was investigated again by Tschopp & Podack (1981), who noticed that isolated C9 when heated to 46 °C underwent spontaneous aggregation to form tubular polymers which the authors called poly-C9. These ringlike structures resembled very closely the ultrastuctural appearance of the extracted complement lesion as published earlier by Bhakdi & Tranum-Jensen (1980). In subsequent reports Podack and his collaborators described this aggregation process in more detail, emphasizing that polymerization could be achieved at 37 °C if the protein was incubated at 1 mg/mL for 64 h (Podack & Tschopp, 1982a), that this process was of functional relevance since the formation of poly-C9 in the presence of liposomes caused marker release from such vesicles (Tschopp et al., 1982), and that the polymerization is a result of a conformational reorganization of the monomeric protein which can be achieved in vitro by heating or in vivo through the functional activity of the C5b-8 complex (Podack et al., 1982).

As reported earlier in preliminary form (Dankert et al., 1983; Shiver et al., 1983) we had been unsuccessful in demonstrating a lytic capacity of the "forming" poly-C9, nor did we observe significant polymerization of purified C9 after incubation at physiologic temperatures. In the present work

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‡ Department of Comparative and Experimental Pathology.
§ Department of Chemistry.

[1] Abbreviations: EA, antibody-sensitized sheep erythrocytes; EAC1–7 and EAC1–8, EA carrying bound complement proteins C1 through C7 or C1 through C8, respectively; MC5b-9, C5b-9 complex assembled on a target membrane; SC5b-9, C5b-9 complex assembled in the fluid phase and containing S protein; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; VB, isotonic veronal buffered saline, containing 5 mM veronal (barbital), 150 mM NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4; GVB, VB containing 0.1% gelatin; TPCK, 1-(tosylamido)-2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 6-CF, 6-carboxyfluorescein; ANS, 8-anilino-1-naphthalenesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Complement proteins are named in accordance with recommendations in *Bull. W. H. O.* (1968).

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we have searched for defined conditions that cause aggregation and have found that monomeric C9 will polymerize (i) when exposed to low concentrations of trypsin, or (ii) when in contact with highly curved lipid bilayers, and (iii) when incubated at temperatures higher than 46 °C and at high (>1 mg/mL) protein concentrations.

Materials and Methods

Chemicals. EDTA,[1] $CaCl_2$, NaCl, KCl, Tris, and sodium barbital were purchased from Fisher Scientific, and MOPS was from Research Organics Inc. Acrylamide, bis(acrylamide), urea, SDS, and dithiothreitol were obtained from Serva Fine Biochemicals, and 6-CF, ANS, and diphenylhexatriene were supplied by Molecular Probes. $Na^{125}I$ was purchased from New England Nuclear and the iodination reagent (Iodo Beads) from Pierce Chemical Co. All other reagents and chemicals were purchased from Sigma. All buffers and solutions were prepared from ultrapure water (Continental Water Systems).

Plasma and Sera. Fresh frozen human ACD-plasma was obtained from Civitan Regional Blood Center (Gainesville, FL), and serum was prepared by addition of $CaCl_2$ to a final concentration of 20 mM and incubation for 2 h at 37 °C.

Antisera Production and IgG Purification. Antisera against human C8 and C9 were raised in goats following standard procedures (Chase, 1967). When tested by immunoelectrophoresis against whole human serum, only a single precipitin line was observed for each antiserum; therefore, we assume that both are monospecific for C8 or C9, respectively. IgG was prepared from 400 mL of antiserum as described by Steinbuch & Audran (1969) with the following modifications. First the antiserum was brought to pH 5.0 by addition of 3 M acetic acid, and then one part of *n*-octanoic acid (Sigma) per 20 parts of serum was added. After being stirred for 30 min at room temperature, the mixture was centrifuged at 30000*g* for 30 min. The pellicle and the pellet were discarded, and the intermediate solution was recovered. An equal volume of saturated ammonium sulfate solution was slowly added. After the solution was allowed to stand for at least 1 h, the precipitate was collected by centrifugation at 30000*g* and the pellet washed 2 times with 50% saturated ammonium sulfate solution and dialyzed against coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 9.5).

Solid-Phase Immunoadsorbent Preparation. Sepharose CL-6B (Sigma) was activated with cyanogen bromide following the method of Nishikawa & Bailon (1975) with the following minor modifications: 25 g of CNBr was dissolved in 25 mL of *N*-methyl-2-pyrrolidone and added dropwise over 5 min to 250 mL of stirred Sepharose beads (in 5 M K_3PO_4, pH 12.5, buffer) kept at <4 °C. After 10 min of continuous stirring, the beads were filtered and washed with 20 volumes of ice-cold water and 20 volumes of ice-cold coupling buffer and then resuspended in 300 mL of the same buffer. Monospecific IgG (anti-C8 or anti-C9) was added to a final concentration of 10 mg/mL beads and coupled overnight at 4 °C with slow tumbling. After filtration and determination of the coupling efficiency, the beads were resuspended in 300 mL of 0.1 M ethanolamine (pH 7.8) to neutralize unreacted groups, then agitated for 2 h, and washed with veronal-buffered saline.

C9 Hemolytic Assay. C8-depleted serum (C8D) was prepared by passage of fresh human serum containing 10 mM EDTA and 0.5 M NaCl through an anti-C8–Sepharose CL-6B column equilibrated with 10 mM EDTA, 10 mM veronal, and 0.5 M NaCl, pH 7.2. The high salt concentration prevents the adsorption of C1q to the antigen–antibody column, and therefore, it is unnecessary to reconstitute the C8D with C1q. The C8D breakthrough fractions were pooled and employed to prepare EAC1–7 as follows. Two milliliters of C8D and 2.5×10^9 EA were incubated for 60 min at 37 °C and then washed 3 times in GVB to remove C9 present in C8D. The EAC1–7 were converted to EAC1–8 by addition of 1.5 μg of purified C8 and incubation at 37 °C for 30 min. After centrifugation and restandardization to 5×10^8 cells/mL, these cells were then used to assay C9 as described by Biesecker & Müller-Eberhard (1980).

Purification of Human C9. Human C9 was isolated from Cohn fraction III (Cutter Laboratories, Berkeley, CA) or from fresh frozen plasma following the general purification scheme of Biesecker & Müller-Eberhard (1980). Because we added several modifications to prevent proteolysis and to speed up the procedure and because results obtained with our C9 preparations were in disagreement with data published by others we describe our purification procedure in more detail. One liter of fresh frozen ACD-plasma was thawed and centrifuged at 3000*g* for 10 min to remove cryoglobulins. Vitamin K dependent enzymes were then precipitated by adding 40 mL of 1 M $BaCl_2$ under stirring for 15 min at 4 °C, and the precipitate was removed by centrifugation at $16000g_{max}$ for 10 min. The supernatant was brought to 7.0% PEG-4000 (Sigma) by slowly adding 500 mL of a 21% aqueous PEG-4000 solution. After the solution was stirred for 15 min and centrifuged at $16000g_{max}$ for 30 min, the pellet was removed and the supernatant brought to 20% by addition of solid PEG-4000. After an additional stirring period of 1 h the suspension was centrifuged at $2500g_{max}$ for 15 min. The pellet containing the C9 activity was dissolved in solubilizing buffer (90 mM NaCl, 10 mM phosphate, and 1 mM PMSF, pH 7.4; conductance <11 $m\Omega^{-1}$) and centrifuged for 15 min at $16000g_{max}$, and the supernatant was applied to a lysine–Sepharose CL-6B column (5 × 16 cm) which was in tandem with a DEAE-Sephacel (2.6 × 40 cm) column. Both columns were equilibrated with solubilizing buffer. All preparations up to this point were performed in plastic vessels. The sample was pumped onto the columns at a flow rate of 100 mL/h followed by elution buffer (=solubilizing buffer without PMSF), and both columns were washed until the absorbance (A_{280}) of the eluent was <0.05. The DEAE-Sephacel column is eluted at a flow rate of 60 mL/h with a linear NaCl concentration gradient formed from 1 L elution buffer and 1 L of buffer adjusted to 30 $m\Omega^{-1}$ with NaCl. Fractions containing C9 activity were analyzed by SDS–PAGE (Laemmli, 1970) and pooled to minimize contaminating proteins. The C9 pool was diluted with 10 mM phosphate (pH 7) to a conductance of 10 $m\Omega^{-1}$ and applied (40 mL/h) to a hydroxylapatite column (1.6 × 12 cm; LKB- HA Ultrogel) equilibrated in 10 mM sodium phosphate, 100 mM NaCl, pH 7.0, first washed with 100 mL of the same buffer and then washed with 50 mM sodium/potassium phosphate, pH 7.8, until A_{280} reached <0.01. The column was then eluted with a linear phosphate gradient formed from 200 mL of 50 mM sodium/potassium phosphate and 200 mL of 300 mM sodium/potassium phosphate at pH 7.8. Fractions containing C9 protein were pooled with the aid of SDS–PAGE analysis, concentrated (Micro-ProDiCon, Bio-Molecular Dynamics), and finally sieved on a Sephacryl S-200 column (2.6 × 75 cm) in 10 mM Tris, 200 mM NaCl, and 0.02% NaN_3, pH 7.2, at a flow rate of 15 mL/h. The C9 usually eluted in about five fractions of 15 mL each, and each fraction was stored separately at −70 °C.

Sometimes we used Cohn fraction III (Cutter Laboratories, Berkeley) as a starting material for C9 isolation. In this case

100 g of Cohn fraction III was solubilized in 1 L of phosphate buffer (10 mM phosphate, 90 mM NaCl, and 1 mM PMSF, pH 7.4), and after removal of insoluble material by centrifugation the solution was further processed by $BaCl_2$ precipitation and plasminogen removal as described above. Although C9 preparations from plasma and from Cohn fraction III had equivalent specific hemolytic activity, one usually could detect by SDS–PAGE analysis some proteolytic degradation in the Cohn preparation. C9 protein concentrations were determined spectrophotometrically by using an extinction coefficient of $E_{280} = 0.96$ mL/(mg cm) (Podack & Tschopp, 1982a).

Proteins. Human C8 was purified as described earlier by Steckel et al. (1982) with the additional $BaCl_2$ precipitation and plasminogen removal steps described above for the C9 purification. We also linked the lysine–Sepharose column directly to the CM-Sepharose column for the first ion-exchange chromatography step. Human α-thrombin was a gift of Dr. John Fenton (Albany, NY), TPCK–trypsin was bought from Worthington, and proteinase K and soybean trypsin inhibitor were from Sigma.

C9 Iodination. C9 was iodinated by the Iodo-Beads (Pierce) method as suggested by the manufacturer. In brief, between 0.1 and 0.5 mg of C9 was incubated with 0.5 mCi of $Na^{125}I$ (New England Nuclear) and two beads (Chloramine T derivatized polystyrene) at 4 °C for 30 min in 10 mM Tris and 145 mM NaCl, pH 7.2, buffer. The iodinated protein was separated from free iodine by gel chromatography on Sephadex G-100 (1 × 5 cm). Specific radioactivity usually ranged between 0.1 and 0.5 μCi/mg.

C9 Proteolysis. Limited proteolysis of monomeric C9 with trypsin was carried out at 37 °C in 10 mM Tris and 200 mM NaCl, pH 7.2, at a C9 concentration of 1 mg/mL and between 2 and 0.1% (w/w) trypsin. The enzyme was diluted from freshly prepared stock solutions of TPCK–trypsin at 10 mg/mL. At different time points aliquots were removed, mixed with twice the weight ratio of soybean trypsin inhibitor, and analyzed for hemolytic activity, aggregation behavior by gel chromatography on a Sepharose 6B column (0.9 × 18 cm), and morphological appearance by electron microscopy. Digestions with 5% (w/w) α-thrombin were carried out in 10 mM Tris, 200 mM NaCl, and 1 mM $CaCl_2$, pH 7.8, as described previously (Ishida et al., 1982).

Lipids. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and egg lecithin were purchased from Avanti (Birmingham, AL) and used without further purification.

Vesicle Formation. Small unilamellar vesicles (SUV) were prepared by drying 40 mg of lipid dissolved in hexane or chloroform–methanol in a test tube under a stream of nitrogen and then lyophilizing the lipid film for 12 h. Four milliliters of buffer (10 mM MOPS, 100 mM KCl, and 0.02% NaN_3, pH 7.0) was added, and the mixture was incubated for 1 h above the phase transition of the lipid. The suspension was then sonicated with a probe sonicator (Heat Systems) under nitrogen for 1 h at 25 °C for egg lecithin and DMPC and at 48 °C for DPPC. The resulting vesicle mixture was centrifuged at $100000g_{max}$ to remove large vesicles and titanium fragments, and the vesicles were used within 1 h after preparation. In the electron microscope the negatively stained vesicles appeared to be unilamellar and of 25–30-nm diameter.

Multilamellar vesicles (MLV) were prepared by hydrating lipid films as above and vortexing at the temperatures indicated for the various phospholipids.

C9–Lipid Interactions. Lipid vesicles (SUV or MLV) at a concentration of 5 μM lipid were incubated at 25 °C in a total volume of 0.4 mL with varying amounts (but in most experiments 50 nM) of monomeric C9 which had been trace labeled with ^{125}I-C9. The mixture was applied to a Sepharose 2B column (1.5 × 30 cm) equilibrated with 10 mM MOPS, 100 mM KCl, pH 7.0, and eluted with the same buffer at a flow rate of 7 mL/h. Fractions were collected and tested for radioactivity and C9 hemolytic activity. Vesicles were detected by forward light scattering at 300 nm in the case of MLV, and SUV were detected by a fluorescence assay described by London & Feigenson (1978). Aliquots (300 μL) of the fractions were made 3 μM in diphenylhexatriene, and the fluorescence was measured in an Aminco-Bowman spectrofluorometer in the ratio mode with excitation at 350 ± 5 nm and emission at 460 ± 5 nm. The lipid concentration was determined from a standard curve as suggested by the authors. The effect of C9 on potassium diffusion potentials was measured with a fluorescence assay (Dankert et al., 1982) using anilinonaphthalenesulfonate (ANS) as a fluorescent probe. ANS fluorescence was measured at 480 ± 5 nm with excitation at 380 ± 5 nm in a temperature-regulated, stirred cuvette with 0.5 mL total volume.

Electron Microscopy. Two different methods were used for specimen preparation. In method A a sample, diluted to about 50 μg/mL in a volatile buffer (0.1 M ammonium acetate and 0.05 M NH_4HCO_3, pH 7.4), was centrifuged against a Formvar carbon-coated specimen grid (400 mesh) in a Beckman airfuge EM-90 rotor at 90 000 rpm. The grid was removed, washed twice in dilution buffer, and floated on a drop of 1% uranyl formate for 1 min and blotted dry. Some samples were prepared by the traditional method (method B) where a sample drop is placed directly on the specimen grid which had been made hydrophilic by application of 0.1% bacitracin. Washing and staining were performed as described above. The grids were imaged at 80 kV in a Zeiss EM-10CR electron microscope and photographed at 50000× direct magnification.

Gel Permeation Chromatography. The aggregation behavior of C9 was analyzed by gel permeation chromatography on Sepharose 6B (0.9 × 18 cm column) using an Isco variable wavelength monitor with a 20-μL flow cell to measure the absorbance of the eluant.

RESULTS

C9 Heat Inactivation. The ability of C9 (at a concentration of 0.4 mg/mL) to spontaneously polymerize upon heating to 46 °C as described by Podack & Tschopp (1982a) was initially analyzed by gel permeation chromatography and hemolytic assays. In contrast to these earlier reports we were unable to detect significant polymerization or loss of hemolytic activity (Shiver et al., 1983). While these experiments were in progress, Podack & Tschopp (1982b) reported in a subsequent paper that the concentration of C9 must be increased to above 1 mg/mL and preferably to as high as 4 mg/mL before efficient aggregation and loss of activity could be observed. In Figure 1 we present our results of incubating C9 at 1 mg/mL under sterile conditions and for different lengths of time. Again, it is evident that C9 at the physiologic temperature of 37 °C will lose not more than 2.5% hemolytic activity even after 300 h of incubation (lower panel, trace A), and as can be demonstrated by gel chromatography (data not shown) about 1–2% aggregated material is formed under these conditions. After the concentration of C9 is increased to 4 mg/mL and C9 is assayed by gel chromatography (Figure 1, upper panel), it becomes apparent that the decrease in monomeric C9 follows closely the loss of hemolytic activity (trace B, lower panel). A steady state of about 60% is reached below which no further decrease can be observed. It is not possible to

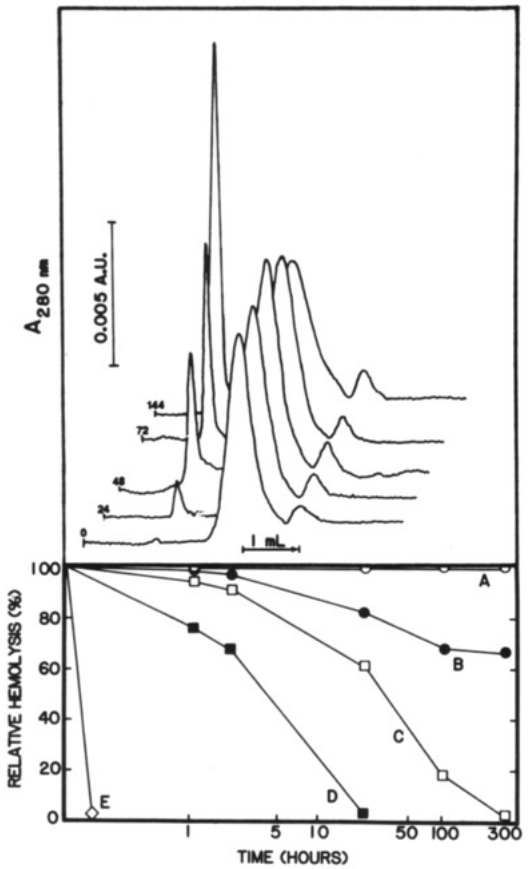

FIGURE 1: Upper panel: time course of C9 aggregation as assayed by gel chromatography. C9 (4 mg/mL in 10 mM Tris, 150 mM NaCl, and 0.02% NaN_3, pH 7.2) was incubated at 37 °C under sterile conditions for 0, 24, 48, 72, and 144 h, and then 2.5-μL aliquots were applied at room temperature to a Sepharose 6B column (0.9 × 18 cm) and eluted at 7 mL/h. Lower panel: loss of C9 hemolytic activity as a function of temperature and concentration. Monomeric C9 in 10 mM Tris, 150 mM NaCl, and 0.02% NaN_3, pH 7.2, was incubated in plastic snap vials under sterile conditions and assayed for hemolytic activity as described under Materials and Methods. Lysis of a control sample kept on ice for the same period as the test sample was set to 100%; maximum hemolysis of the control sample was 66% of water lysis. Line A, C9 = 1 mg/mL, T = 37 °C; line B, C9 = 4 mg/mL, T = 37 °C; line C, C9 = 1 mg/mL, T = 46 °C; line D, C9 = 1 mg/mL, T = 48 °C; line E, C9 = 1 mg/mL, T = 56 °C.

directly compare the decrease of the monomer peak with the increase of the aggregate peak since the latter is artificially increased because light scattering artifacts contribute to the apparent absorbance. Incubation of C9 at higher temperatures (46, 48, and 56 °C) for prolonged times will lead to loss of hemolytic activity (traces C–E, lower panel) and aggregation similar to the one shown in Figure 1 (upper panel). Analysis of the protein at the end of the incubation period by SDS–PAGE did not reveal any significant proteolysis or formation of SDS-resistant polymers (data not shown). Although most of these experiments were done in Tris buffer, we have also performed similar experiments using MOPS or phosphate buffers and have varied the salt concentration between 0.1 and 0.2 M without observing significant differences.

The aggregated material produced by heating as described above was imaged in the electron microscope. To achieve a representative documentation of *all* structures present in these samples, we centrifuged aliquots of the different incubation mixtures against a grid in the airfuge-EM rotor and then processed the deposited protein for negative staining by conventional methods. Heating of C9 for 2 h at 48 °C (Figure 2B), or for 5 min at 56 °C (Figure 2C), produces C9 aggregates that are predominantly in a stringlike form. In contrast

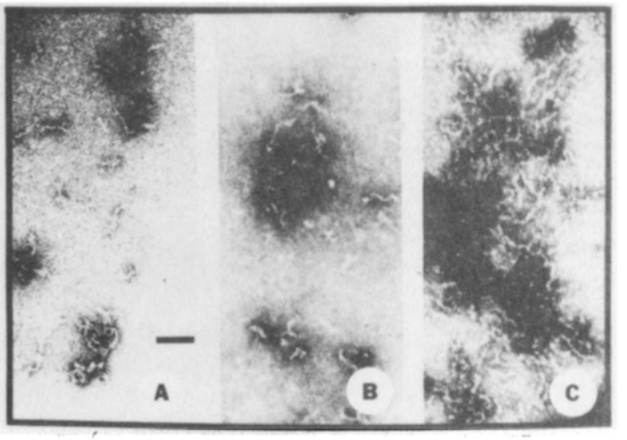

FIGURE 2: Ultrastructure of heated C9. C9 was incubated at 1 mg/mL as described in the legend to Figure 1, and aliquots were centrifuged in the airfuge-EM rotor and prepared for electron microscopy as described under Materials and Methods. Panel A, C9 after incubation for 300 h at 37 °C; panel B, 3 h at 48 °C; panel C, 5 min at 56 °C. Scale bar = 50 nm.

to the report of Podack & Tschopp (1982a), who noticed predominantly tubular structures (poly-C9) in their heated C9 samples, we could only very occasionally observe tubular poly-C9 at 37 or 48 °C and never at 56 °C, and it did not matter whether the total material in the incubation mixture or only the high molecular weight fraction obtained after gel chromatography was imaged. Inspection of the C9 sample incubated for 300 h at 37 °C in the electron microscope (Figure 2A) reveals the occasional presence of some aggregated material. It is impossible to determine from electron micrographs the concentration of the aggregates since the monomeric protein cannot be quantitated by this method; however, on the basis of gel chromatography we cannot detect more than 1–2% aggregated material in the 37 °C sample (see above). When the more concentrated (4 mg/mL) C9 sample after incubation for 300 h was observed in the electron microscope, the pictures resembled the one shown in Figure 2A except that the frequency of finding aggregates was increased.

Enzymatic Proteolysis. Because of our inability to reproduce the formation of tubular poly-C9, we searched for differences and procedures that could explain the discrepancies between our results and those of Podack and co-workers. Although we carefully avoid proteolysis during the purification of C9 (see Materials and Methods), we tested whether a contaminating protease could aid in formation of poly-C9. We noticed to our surprise that low concentrations of trypsin will trigger the formation of tubular poly-C9 from monomeric C9 during incubation at 37 °C and that polymerization is independent of C9 concentration. The presence of poly-C9 rings can be visualized in the electron microscope after 5 min of incubation (Figure 3B) although some stringlike material is also visible. The latter aggregates are completely absent when samples taken at later time points are imaged (Figure 3C,D). The resulting poly-C9 ring (see insert in panel D) appears indistinguishable from the earlier published poly-C9 structures that were apparently produced by heating (Podack & Tschopp, 1982a). Analysis of this proteolysis reaction by gel chromatography confirms the ultrastructural observations. A high molecular weight, strongly light-scattering peak is observed after 18 min of incubation (Figure 4, trace B). In contrast to the heating experiments described above, however, it is also evident that the C9 monomer peak decreases with concomitant appearance of a low molecular weight material which most probably represents C9 fragments. The experiments shown here were done with 1% (w/w) trypsin, but similar results were

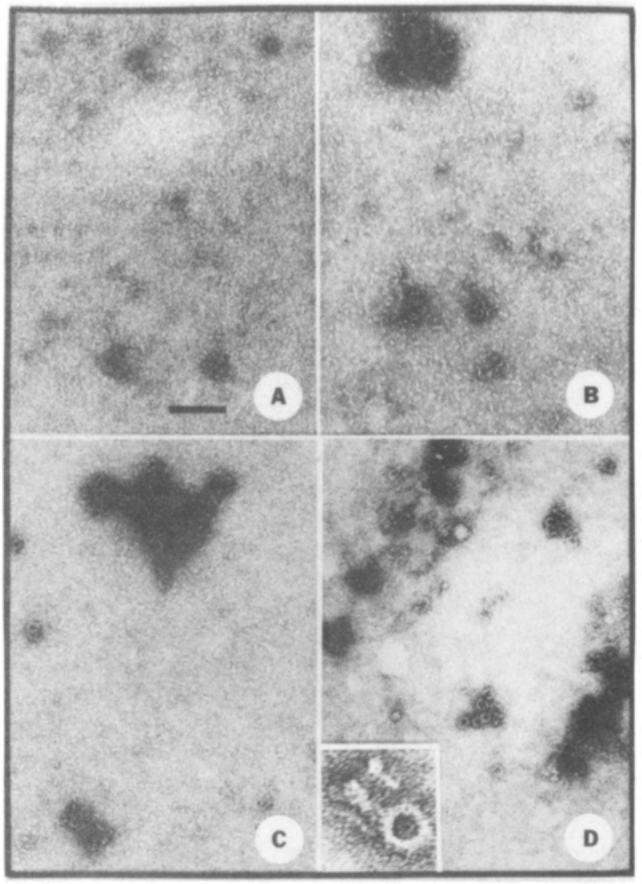

FIGURE 3: Ultrastructure of trypsinized C9. Monomeric C9 (1 mg/mL) was incubated at 37 °C with 1% (w/w) TPCK–trypsin, and 20-μL aliquots were withdrawn at different time points (panel A, t = 0 min; panel B, t = 5 min; panel C, t = 25 min; panel D, t = 60 min), negatively stained with 1% uranyl formate, and imaged in the electron microscope. Scale bar = 100 nm. Insert panel D: top view and side view of poly-C9 produced by trypsinization; scale bar = 18 nm.

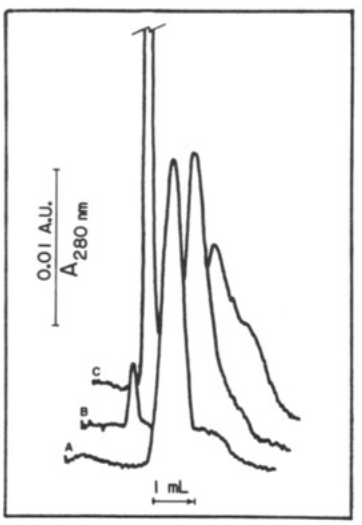

FIGURE 4: Gel chromatography of trypsinized C9. Trypsinization was carried out as described in the legend to Figure 3, and 20-μL aliquots were withdrawn at t = 0 min (trace A), t = 18 min (trace B), and t = 90 min (trace C), twice the weight of soy bean trypsin inhibitor was added, and the mixture was analyzed on Sepharose 6B as described in the legend to Figure 1.

observed at 10-fold lower trypsin concentrations albeit at a somewhat slower rate and with less degradation of monomeric C9. Inclusion of soybean trypsin inhibitor at twice the concentration of trypsin abrogated C9 polymerization and digestion. High concentrations of trypsin (10% w/w) produced only fragments and no poly-C9.

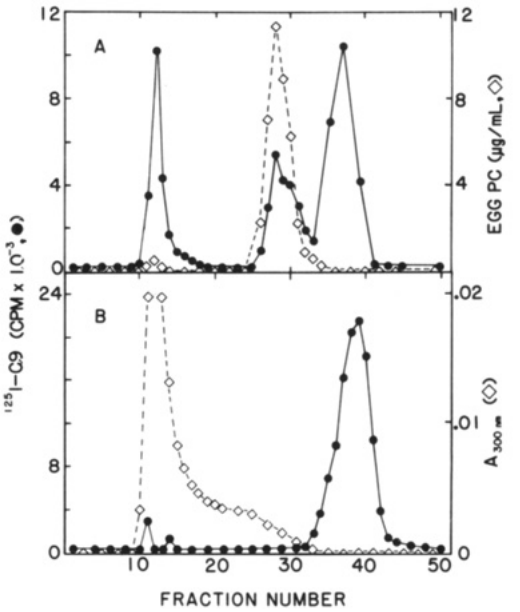

FIGURE 5: Interaction of monomeric C9 with lipid vesicles. Monomeric, ^{125}I-trace-labeled C9 was incubated with SUV prepared from DPPC (panel A) or with MLV prepared from egg lecithin (panel B), and the mixtures were chromatographed on Sepharose 2B as described under Materials and Methods. Fractions (1.6 mL) were analyzed for radioactivity (●) and for vesicle content (◇) by forward light scattering at 300 nm in the case of MLV or by determining the lipid concentration in the case of SUV (see Materials and Methods).

As shown in Figure 1 when monomeric C9 at a concentration of 4 mg/mL is incubated at 37 °C, the hemolytic activity declines with time until a steady state of about 60% residual activity is reached. To test whether this represents a population of C9 that is resistant to aggregation per se, we separated the 40% aggregated fraction by differential ultracentrifugation and incubated the remaining monomeric C9 at 37 °C with 0.2% (w/w) trypsin for 12 h. Gel chromatography of the reaction mixture demonstrated the susceptibility of this monomeric C9 fraction to trypsin-induced aggregation (data not shown), indicating that heat and trypsin induced aggregation may be different processes. Thus far we have only tested trypsin, α-thrombin, and proteinase K with respect to their effect on C9 aggregation and found that thrombin cleaves C9 into two fragments without demonstrable polymerization, while proteinase K digests the protein into small fragments, and again no polymerization is detectable. We also noticed that the decline in hemolytic activity of the more concentrated C9 sample (Figure 1, trace B) after 100 h could not be prevented by inclusion of soybean trypsin inhibitor or PMSF in the incubation mixture (data not shown).

C9 Interaction with Lipid Bilayers. In their original report Tschopp & Podack (1981) described that the forming poly-C9 would interact with small unilamellar vesicles (SUV) and cause marker release from such liposomes. Since it was conceivable that exposure of monomeric C9 to lipids could enhance the formation of poly-C9, the interaction of monomeric C9 with lipid bilayers was analyzed by gel chromatography on Sepharose 2B. Monomeric, ^{125}I-trace-labeled C9 was incubated with SUV (prepared from DPPC) at a molar ratio of 100 lipid molecules per one C9 and at 23 °C, well below the phase transition temperature of this lipid. After 5 min the sample was applied and the column eluted with buffer. When the fractions were assayed for ^{125}I-C9, a significant portion of the label was found in the void volume of the column (Figure 5A,

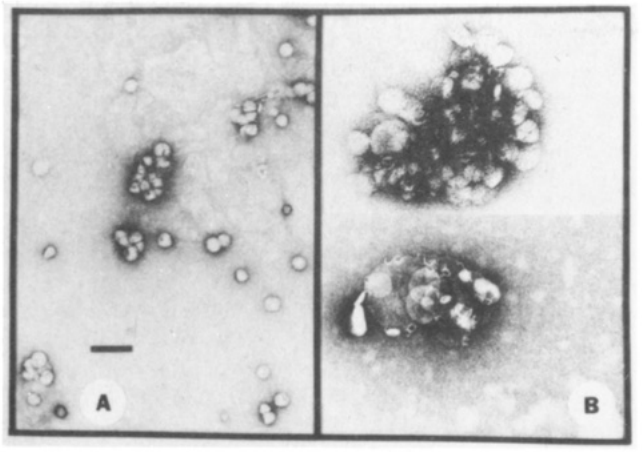

FIGURE 6: Ultrastructure of vesicle-bound C9. Aliquots of fraction 28 (panel A) or fraction 12 (panel B) from the chromatogram shown in Figure 5A were processed for electron microscopy and imaged. Scale bar = 100 nm.

fraction 12) together with a minute amount of lipid vesicles, and about 25% of the iodinated C9 elutes together with SUV (fraction 28). Control experiments showed that SUV alone also elute with a peak in fraction 28. When fraction 28 of Figure 5A was examined in the electron microscope, vesicles could be detected which were indistinguishable from control SUV (Figure 6A). When fraction 12 of Figure 5A was imaged, however, large vesicle aggregates could be seen (Figure 6B) resembling a cluster of grapes. Some vesicles appear 2–3 times enlarged compared to the original SUV. In addition protein aggregates are clearly visible within the multivesicular aggregates, with some of them displaying the distinct tubular structure of poly-C9.

When vesicles were prepared from egg lecithin or DMPC instead of DPPC and incubated as described above, no difference was observed, which indicated that monomeric C9 interacts with SUV independent of the phase state of the lipid. This interaction of C9 with SUV is in marked contrast to its behavior when exposed to multilamellar lipid vesicles (MLV). In this case no interaction can be detected; MLV elute in the void volume of the Sepharose 2B without bound C9 (Figure 5B), and no protein aggregates can be detected in this fraction in the electron microscope (data not shown). The minute amount of radioactive high molecular weight material in fraction 12 does not represent MLV-bound C9 because it can be detected in control chromatograms without MLV.

The ability of C9 to interact with SUV prompted us to investigate the nature of the interaction. According to one postulate (Podack & Tschopp, 1982a) vesicles containing poly-C9 are damaged because of the insertion of a large water-filled channel across the lipid bilayer. Thus, the permeability of C9-treated SUV was tested with 6-carboxyfluorescein (6-CF) by preparing vesicles in the presence of a self-quenching concentration of 6-CF (100 mM) as described by these authors. Unencapsulated fluorophore was separated on Sephadex G-100. Vesicles at a concentration of 5 μM lipid were monitored for fluorescence, and monomeric C9 was added to a final molar ratio of 100 to 1 (lipid to C9) directly into the fluorometer cuvette. No significant increase in fluorescence could be observed at 23 °C (data not shown). Fractionation of those vesicles that had been incubated with 50 nM C9 for 5 min at 23 °C on Sepharose 2B again yielded two populations of vesicles carrying C9 (data not shown) as was demonstrated above (Figure 5A) for SUV without 6-CF. Fractions were tested for encapsulated 6-CF by addition of Triton X-100, and as shown in Figure 7 both populations release the fluorophore,

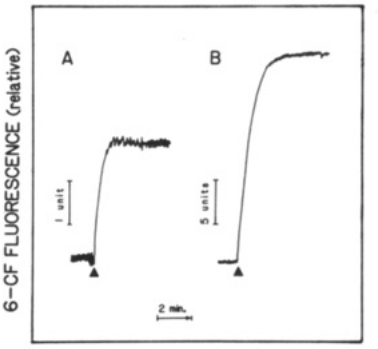

FIGURE 7: Release of 6-carboxyfluorescein from lipid vesicles. Gel chromatography of SUV (prepared from DPPC) containing 100 mM 6-CF was performed as in Figure 5A. A 300-μL sample was added to a stirred cuvette in the fluorometer, and the fluorescence was continuously monitored at 520 ± 4 nm (excitation = 480 ± 4 nm). At the indicated time (▲) Triton X-100 was added to a final concentration of 0.1%. Trace A shows fluorescence of an aliquot of fraction 12 and trace B from an aliquot of fraction 28.

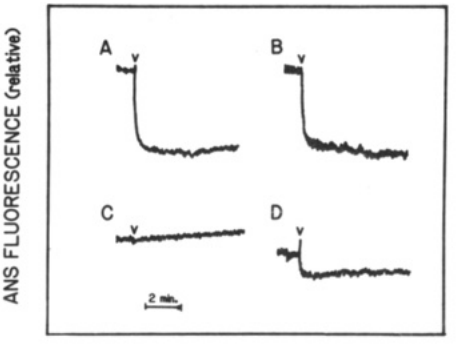

FIGURE 8: Generation of a potassium diffusion potential in vesicles with bound C9. Chromatography of SUV was performed as in Figure 5A with vesicles prepared from egg lecithin. A 20-μL aliquot of a collected fraction was diluted into 280 μL of 10 mM MOPS and 100 mM choline chloride, pH 7.0, in a stirred fluorometer cuvette. ANS (5 μM) was added, and the fluorescence at 480 ± 4 nm was continuously monitored (excitation = 380 ± 4 nm). At the indicated times (v) valinomycin was added to 0.1 μM. Trace A, fraction 28 of a sample of SUV incubated without C9 and then chromatographed as shown in Figure 5A; trace B, same as trace A except that the SUV were incubated with 1 mol % C9 prior to chromatography; trace C, same as trace A except that the SUV sample was diluted into 10 mM MOPS and 100 mM KCl, pH 7.0; trace D, fraction 12 of a sample of SUV incubated with 1 mol % C9 and chromatographed as shown in Figure 5A.

indicating that their integrity was not impaired by the presence of C9 even when the latter was polymerized.

Lipid bilayers are highly impermeable to ions, and therefore, a more stringent test is to measure the capacity of vesicles to hold a membrane diffusion potential. Since both vesicle populations contained 100 mM KCl, they were diluted into isoionic but potassium-free buffer, and the ionophore valinomycin was used to set up a potassium diffusion potential as described earlier (Dankert et al., 1982). Diffusion potentials can be measured with certain fluorescent dyes such as carbocyanines or anilinonaphthalenesulfonic acid (ANS) (Sims et al., 1974). As demonstrated in Figure 8 both vesicle populations are impermeant to potassium because addition of valinomycin leads to a decrease in fluorescence. This is to be expected because of the formation of a trans-negative membrane potential which causes the negatively charged dye to be repelled from the bilayer leading to a decrease in its fluorescence. If a potassium gradient does not exist, then the addition of valinomycin does not lead to a decrease in fluorescence, as is shown in Figure 8C for SUV diluted into a buffer containing the same concentration of potassium as

the vesicle lumen. The change in fluorescence is of course much smaller for the poly-C9 aggregated vesicles because much fewer vesicles are present in this fraction (Figure 5A). However, it is important to note that even poly-C9 is ineffective in causing ion leaks across vesicle membranes. Thus, we have no evidence that monomeric C9, aggregated C9, or a forming poly-C9 is membranolytic at reasonable lipid to protein concentrations.

Discussion

When human C9 was originally isolated by Hadding and Müller-Eberhard (1969), these investigators noticed that the protein was very sensitive to heating; half of the hemolytic activity was lost within 5 min at 56 °C. The cause for this inactivation is the formation of high molecular weight aggregates with concomitant loss of monomeric C9. Inspection of these aggregates in the electron microscope revealed the presence of stringlike structures (Figure 2) which resemble those published earlier (Podack & Tschopp, 1982a) for aggregates produced by denaturation of C9 with guanidine hydrochloride. These investigators also presented data that indicated that C9 at a concentration of 0.4 mg/mL could be aggregated to form tubular polymers, which they called poly-C9, if the incubation temperature was decreased to 37 °C and the time increased to 64 h. In their hands formation of poly-C9 paralleled the loss of hemolytic activity. However, we were unable to confirm these results. Incubation of C9 at 37 °C for 300 h at physiologic ionic strength and sterile conditions caused not more than 2% loss in hemolytic activity, nor could we detect significant formation of aggregated material. At higher temperatures (46 and 48 °C) aggregation was noticed after several hours incubation, but the aggregated material was predominantly stringlike and only occasionally could we detect tubular poly-C9. Because of the perceived importance of this circular polymerization reaction to the molecular architecture of the membrane attack complex (MC5b-9) and the function of the complement lesion (Podack et al., 1982), we searched for experimental conditions that could explain these discrepancies but were not successful. Although increasing the protein concentration during incubation to 4 mg/mL resulted in some loss of hemolytic activity, the reaction is by no means complete; even after 300 h of incubation 60% of the protein is still active. In retrospect it is not too surprising that C9 should be stable at 37 °C and physiologic ionic strength since it circulates in plasma under these conditions.

Isolation of intact proteins from plasma is very often difficult because of the presence of large amounts of potential proteases, and C9 is no exception although its activity is not very sensitive to partial proteolysis (Biesecker et al., 1982). For this reason we considered the possibility that either a contaminating protease in the final product or the presence of proteolytically altered C9 could explain the differences between our results and those of Podack and associates. Indeed, short-time trypsinization of C9 at a low enzyme concentration proved to be an effective means of inducing aggregation of C9, but more importantly only the tubular form, or poly-C9, is produced. None of the stringlike material which is so dominant in heat or guanidine-denatured C9 can be detected. Poly-C9 produced by trypsinization is resistant to SDS dissociation and must be of very high molecular weight since it does not enter a 2.5% polyacrylamide gel (data not shown). Morgan et al. (1983) also suggested that proteolysis may be involved in aggregation of C9. In our hands, the polymerization reaction can be inhibited by soybean trypsin inhibitor, and it appears that a specific peptide bond must be cleaved because cleavage of C9 at a different position by α-thrombin is ineffective in producing poly-C9. We are now in the process of finding conditions to depolymerize trypsin-produced poly-C9 in order to identify the nature of the proteolytic modification that leads to self-aggregation in such a defined manner.

A second discrepancy to the earlier reports was noticed when we investigated the interaction of C9 with lipid bilayers. Tschopp et al. (1982) had indicated that only the forming poly-C9 complex was capable of interacting with lipid vesicles. Our data indicate, however, that monomeric C9 at low concentrations associates immediately (<5 min) with SUV even at a temperature (21 °C) at which no poly-C9 formation was ever reported in the absence of other external agents. This interaction is sufficiently strong to withstand gel chromatography, but more importantly it also leads to C9 aggregation and, as demonstrated by electron microscopy, to formation of tubular poly-C9 in addition to stringlike products. It is interesting to note that about half of the protein that interacts with SUV forms aggregates and that these polymers interact with SUV to form large structures that resemble clusters of grapes. The other half also remains with the vesicles after sieving, and because the elution position of the SUV is unchanged, we assume that C9 is bound as monomers or perhaps low multimers. We conclude from these results that C9 polymerization in the presence of lipids is not temperature driven but is effected by protein-lipid interactions. However, such interactions require the presence of highly curved bilayers because C9 does not interact with large MLV or flat, solvent-free, bimolecular lipid membranes (BLM) (Esser et al., 1984). We propose that a hydrophobic domain in native C9 (Biesecker et al., 1982; Ishida et al., 1983) associates with lipids, and if the packing density of the lipid headgroups is low—as is the case in highly curved bilayers—then the protein will intercalate. At this point, however, we have no good explanation as to why a significant number of molecules aggregate and others remain dispersed in the vesicle membrane. At the lipid to C9 ratios at which most of our experiments were conducted there are about 50 C9 monomers per SUV. It has been proposed that about 15 ± 3 molecules comprise a tubular poly-C9 (Tschopp et al., 1984), and on the basis of our electron microscope images, we assume that the stringlike aggregates contain at least as many C9 monomers. Therfore, unless the interaction of C9 with SUV is cooperative, one would expect that there should be an average of 50 C9 monomers or about 3–4 poly-C9 per vesicle; however, this was clearly not the case, and further studies are required to obtain a satisfactory explanation.

A third important result, which is also not in agreement with the earlier reports, is our demonstration that the association of C9, either in the aggregated or in the dispersed form, with SUV does not lead to membrane impairment. Tschopp & Podack (1981) had postulated that "C9 alone is a cytotoxic molecule" because they observed release of a trapped fluorescent marker from SUV upon interaction with C9. As we have pointed out earlier (Dankert et al., 1983) and have demonstrated here in more detail, marker release cannot be observed at C9 to vesicle ratios at which the C9 molecules associate in a stable form with vesicles. The vesicle membrane is not even permeant to small potassium ions because diffusion potentials can still be created across the lipid bilayers. This result is equally true for those vesicles that associate in form of clusters with C9 polymers and for those that carry unaggregated C9. Tschopp et al. (1982) used a C9 to lipid molar ratio of 1 to 8 in order to demonstrate release of 6-CF from vesicles (see Figure 3 of their report), but at such high con-

centrations many other soluble proteins will cause membrane damage. Furthermore, the same group has recently reported that each poly-C9 tubule apparently can bind about 720 molecules of deoxycholate (Tschopp et al., 1984). If half of the monomeric C9 forms poly-C9 and if each poly-C9 binds similar quantities of lipids, then not a single vesicle will remain intact at such a high protein concentration.

The major question that needs to be asked at this point is how relevant is the observed polymerization to the mode of action of C9? There are two types of aggregation products, stringlike polymers and tubular polymers, or poly-C9. Podack & Tschopp (1982a) argued that poly-C9 is part of the MC5b-9 complex and because of its structure produces the observed enhancement of the rate of hemolysis compared to the much slower EAC1–8 lysis. If this view is correct, then one could propose that the stringlike C9 aggregates are similar to other protein aggregates that are generally formed by heat denaturation. Furthermore, the crucial step in development of the lytic function would then be the formation of poly-C9. Both pathways of poly-C9 formation that we have described here could conceivably function in vivo. The intermediate C5b-8 complex could possess intrinsic proteolytic activity, and the report by Kolb et al. (1982) that C6 may be a serine esterase increases the validity of this speculation. These authors found that the hemolytic activity of C6 was susceptible to serine esterase inhibitors and the protein cleaved synthetic substrates. The natural substrate for the C6 enzymatic activity was not described, and it is tempting to speculate that C9 might play this role. The likelihood of this possibility becoming reality, however, is diminished by the inability of others (DiScipio & Gagnon, 1982) to confirm the results of Kolb and co-workers. The second reaction pathway that leads to poly-C9 formation that we have detected could also function in vivo. As was shown by one of us and discussed in more detail earlier (Esser et al., 1979; Esser, 1982), the C5b-8 complex is very effective in reorienting lipid bilayers with ordered fatty acid chains into a disordered structure. Because monomeric C9 does not associate with flat bilayers but interacts with highly curved lipid vesicles, one could imagine that those lipids in the vicinity of the C5b-8 complex which are less densely packed allow the protein to penetrate and bind more firmly. Both of these mechanisms do not require that C9 remains tightly accociated with the C5b-8 complex in the membrane, and thereby, one could explain the finding of Monahan et al. (1983) that C9 cannot be chemically cross-linked to C8 in the C5b-8 complex on the target membrane. Before we can be sure, however, that poly-C9 formation parallels enhancement of lysis, the exact stoichiometry of the MC5b-9 complex must be known. Sims (1983) and Stewart et al. (1984) have clearly shown that the ratio of C9 to C8 on target membranes never exceeds 4 to 1, and Ware & Kolb (1981) demonstrated that in the extracted MC5b-9 complex this ratio is about 6 to 1. Thus, if 15 ± 3 C9 molecules are required for poly-C9 formation, then at unlimited C9 input one should observe an uptake of 15 C9 molecules per 1 C8 on a target membrane. However, neither Sims nor Stewart et al. were able to observe such high C9 uptake, and at this point it is difficult to decide why the poly-C9 stoichiometry does not agree with the MC5b-9 stoichiometry. If both numbers are correct, then perhaps poly-C9 is not part of the lytic complex and is formed separately as a side product. At this time we actively pursue the latter explanation because as we report elsewhere (Dankert & Esser, 1985) α-thrombin-cleaved C9 is hemolytically completely active, but it cannot form tubular rings under the conditions described here. When such modified C9 is used to reconstitute C9-depleted serum and this serum is then used in hemolysis experiments, we cannot detect in the electron microscope any evidence for the formation of the typical complement lesion on the target membrane, indicating that this particular polymerization product is not required for function.

In any case, there appears to be no disagreement with respect to the conclusion that once C9 is polymerized it is hemolytically inactive, and it does not matter whether tubular or stringlike aggregates are formed. Our results also indicate that aggregated C9 is not membranolytic in general. As a working hypothesis we are considering that polymerization of C9 may actually serve to inactivate "active" C9, although at the present time we do not know what constitutes an active C9 molecule. However, because all other reactions in the complement pathways are tightly controlled in order to protect innocent bystander cells, it does not seem unreasonable to suggest that some kind of a control reaction should also function at this stage.

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Registry No. C9, 80295-59-6; DMPC, 13699-48-4; DPPC, 2644-64-6; trypsin, 9002-07-7.

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Use of Virus-Attached Antibodies or Insulin Molecules To Mediate Fusion between Sendai Virus Envelopes and Neuraminidase-Treated Cells†

A. G. Gitman,‡ I. Kahane,§ and A. Loyter*,‡

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel, and Department of Membrane and Ultrastructure Research, Hadassah Hospital-Hebrew University, Ein Karem, Jerusalem, Israel

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ABSTRACT: Anti-human erythrocyte antibodies or insulin molecules were covalently coupled to the glycoproteins (the hemagglutinin/neuraminidase and the fusion polypeptides) of Sendai virus envelopes with *N*-succinimidyl 3-(2-pyridyldithio)propionate and succinimidyl 4-(*p*-maleimidophenyl)butyrate as cross-linking reagents. Reconstituted Sendai virus envelopes, bearing covalently attached anti-human erythrocyte antibodies or insulin molecules, were able to bind to but not fuse with virus receptor depleted human erythrocytes (neuraminidase-treated human erythrocytes). Only coreconstitution of Sendai virus glycoproteins, bearing attached anti-human erythrocyte antibodies or insulin molecules with intact, untreated viral glycoproteins, led to the formation of fusogenic, targeted reconstituted Sendai virus envelopes. Binding and fusion of reconstituted Sendai virus envelopes, bearing anti-human erythrocyte antibodies or insulin molecules, with neuraminidase-treated human erythrocytes were blocked by the monovalent fraction, obtained after papain digestion of immunoglobulins, made of anti-human erythrocyte antibodies or free insulin molecules, respectively. The results of this work demonstrate an active role of the viral binding protein (hemagglutinin/neuraminidase polypeptide) in the virus membrane fusion process and show a novel and efficient method for the construction of targeted, fusogenic Sendai virus envelopes.

Resealed vesicles made of pure phospholipid molecules (liposomes) (Papahadjopoulos et al., 1982) or fusogenic reconstituted Sendai virus envelopes (RSVE)[1] (Loyter & Volsky, 1982) have been used during the past few years as an efficient biological carrier for the introduction of otherwise impermeable molecules into living cells. RSVE have been also shown to be powerful vehicles for the implantation of various membrane components such as virus, lectin, or hormone receptors into

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‡ Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem.

§ Department of Membrane and Ultrastructure Research, Hadassah Hospital-Hebrew University.

[1] Abbreviations: IgG, immunoglobulin G; F(ab), monovalent fraction obtained after papain digestion of IgG; RSVE, reconstituted Sendai virus envelopes; HE, human erythrocytes; PMSF, phenylmethanesulfonyl fluoride; anti-HE, anti-human erythrocyte antibodies; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; DTT, dithiothreitol; HN, hemagglutinin/neuraminidase glycoprotein of Sendai virus; F, fusion factor; polypeptide–MPB, polypeptide–(maleimidophenyl)butyrate; polypeptide–PDP, polypeptide–(pyridyldithio)propionate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; anti-HE-F(ab), F(ab) fraction of anti-human erythrocyte antibodies; Tris, tris(hydroxymethyl)aminomethane.

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