Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6426–6430, October 1981
Immunology

Assembly of the functional membrane attack complex of human complement: Formation of disulfide-linked C9 dimers

(immune lysis/membrane channel heterogeneity/covalent protein-protein interactions/immunoreplication procedures)

CARL F. WARE AND WILLIAM P. KOLB

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284

Communicated by Frederic C. Bartter, July 9, 1981

ABSTRACT **The 158,000 M_r protein, previously designated C5c, present in fully assembled complement (C) membrane attack complexes (MC5b-9) has been identified as a disulfide-bonded dimer of C9. This conclusion was based on the observations that: (*i*) a portion of the ^{125}I-radiolabeled precursor C9 incorporated into MC5b-9 complexes comigrated with the 158,000 M_r protein band in NaDodSO$_4$/polyacrylamide slab gels; (*ii*) monospecific antisera produced against native C9 and the 158,000 M_r protein immunologically crossreacted with monomeric native C9 by double immunodiffusion and with monomeric C9 and the 158,000 M_r protein on immunoreplication procedures; and (*iii*) two-dimensional NaDodSO$_4$/polyacrylamide slab gel electrophoresis, in which the second dimension was conducted under reducing conditions, revealed that the 158,000 M_r protein contained two identical 71,000 M_r subunits which comigrated with monomeric C9. Molar ratio estimates indicated that 1 mol of C5b, C9 dimer, C6, C7, and C8 and 3–4 mol of C9 monomer were present per MC5b-9 complex. Each fully assembled membrane-bound MC5b-9 complex would therefore have a calculated M_r of 982,000. The presence of C9 dimers in the hemolytically active 29S dimeric form of the MC5b-9 complex and the absence of C9 dimers in the hemolytically inactive 23S monomeric form of the fluid phase SC5b-9 complex strongly suggest an important role for C9 dimer formation in MC5b-9 complex structure and function. The most probable function of C9 dimers would be the formation of intercomplex disulfide crosslinks which would provide a mechanism to stabilize the assembly of MC5b-9 into aggregates of increasing size on the target membrane surface which would thus be responsible for the observed pore size heterogeneity of functional C lesions.**

Complement (C) is a sequential, multimolecular system of plasma proteins which can be activated by various immunological as well as nonimmunological stimuli (1). C activation, which can proceed via either the classical (2) or the alternative pathway (3), is achieved through a series of cascading reaction steps that are dependent upon the conversion of serum zymogens to active serine esterase enzymes (4, 5). Activation of either C pathway results in the expression of multiple biological activities which are mediated through specific membrane binding events with subsequent modulation of various membrane functions.

One of the major membrane-modulating functions of C, the damage to biological membranes associated with C-dependent cytolysis, is effected by the C5b-9 membrane attack complex (MC5b-9) (6–9). MC5b-9 complex assembly is initiated by the limited proteolytic cleavage of C5 to C5b and C5a by the C5 convertase enzymes of either C pathway (10). Nascent C5b combines with C6 and C7, resulting in the formation of a C5b67 trimolecular complex (7, 11) capable of binding phospholipids which results in the firm attachment of this intermediate complex to the target membrane surface (12). Binding of C8 and C9 to MC5b-7 complexes dramatically increases the expression of hydrophobic binding sites within the MC5b-9 proteins, enabling the complex to become at least partially embedded within the membrane interior (9, 12–14). As a result of this hydrophobic insertion, the MC5b-9 complexes behave as integral membrane components requiring detergent extraction for solubilization (15, 16).

Detergent-solubilized MC5b-9 complexes obtained from C-lysed membranes constitute a heterogeneous population of oligomeric structures (16). Different investigators have reported widely divergent properties for detergent-solubilized MC5b-9 complex populations—i.e., the major component has been reported to represent a 1.7×10^6 M_r MC5b-9 dimer (17), a 1.9×10^6 M_r MC5b-9 dimer (16), and a 1×10^6 M_r MC5b-9 monomer (18) having sedimentation coefficients of 33.5, 29, and 26 S, respectively. However, careful examination of currently available data (16–19), particularly the recent report of Podack and Müller-Eberhard (19), indicates that the major membrane bound form of the complement attack complex is a MC5b-9 complex dimer. This ability of MC5b-9 complexes to form multimeric aggregates (16–18) suggests a molecular basis for the observed enlargement of the average functional C lesion on a target cell surface with increasing multiplicity of membrane-bound MC5b-9 complexes (16, 20–22).

Our previous studies concerning the subcomponent composition of the membrane attack complex of human C indicated that an unidentified component, termed X protein, was incorporated into fully assembled MC5b-9 complexes obtained from detergent-solubilized C-lysed biological membranes (16). The X protein migrated on NaDodSO$_4$/polyacrylamide gel electrophoresis under nonreducing conditions as a 158,000 M_r protein that was unrelated to C5 (16).

We now report that the 158,000 M_r protein represents a covalently bonded C9 dimer that is formed during MC5b-9 complex assembly. The significance of C9 dimer formation to membrane attack complex structure and function is discussed.

MATERIALS AND METHODS

Purified C Proteins. Highly purified C3 (23), C5 (24), C6 (25), C7 (25), C8 (26), and C9 (27) were isolated as described. Purified C proteins were radiolabeled with Na^{125}I by the solid-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Nomenclature for complement proteins follows that set forth by the World Health Organization [(1968) *Bull. WHO* **39**, 935]. C, complement; MC5b-9, C5b-9 complexes assembled on erythrocyte membranes; SC5b-9, C5b-9 complexes containing S protein assembled upon C activation of serum; E, sheep erythrocyte(s); EA, antibody-sensitized sheep erythrocytes; EAC, C-lysed EA membranes.

phase glucose oxidase/lactoperoxidase system (16) (Enzymobeads, Bio-Rad). Specific activities of ^{125}I-radiolabeled C proteins were 0.2–1 × 10^6 cpm/μg with retention of a minimum of 80% specific hemolytic activity.

MC5b-9 Complex Purification. MC5b-9 complexes were isolated in highly purified form from C-lysed sheep erythrocyte (E) membranes as described (16). Briefly, 2 × 10^{11} antibody-sensitized sheep E (EA) were incubated for 60 min at 37°C with 250 ml of freshly drawn normal human serum containing 1 × 10^7 cpm of the radioiodinated C component of interest. C-lysed EA membranes (EAC) were washed three times in 5 mM Na borate, pH 8.8/10 mM EDTA/1 mM phenylmethylsulfonyl fluoride (BEP buffer) by centrifugation at 27,000 × g for 20 min. Washed EAC were solubilized by addition of 1.0% zwitterionic detergent, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB_{12}; Calbiochem–Behring) in BEP buffer at a final detergent-to-protein ratio of 10:1 (wt/wt). The detergent extract was cleared of membrane debris by centrifugation at 27,000 × g for 30 min, concentrated by ultrafiltration, and applied to a Bio-Gel A-50m column (1.5 × 120 cm) equilibrated with BEP buffer containing 0.15% SB_{12} and eluted at 4 ml/hr. The MC5b-9 elution profiles were monitored for incorporation of radioiodinated C precursor proteins, and subunit composition was visualized by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (28). Fractions containing the 1.9 × 10^6 M_r MC5b-9 dimer complex (16) were pooled, concentrated by ultrafiltration, and stored at 1°C. These pools contained approximately 80% 29S MC5b-9 dimer and 15–20% 34S or larger MC5b-9 aggregates as determined by sucrose density gradient ultracentrifugation (16).

Polyacrylamide Gel Electrophoresis. Polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ was conducted as described by Laemmli (28). Protein samples were denatured in 50 mM Tris/10 mM phosphate, pH 6.8/4% $NaDodSO_4$/2 mM EDTA/10 mM iodoacetamide/5% (vol/vol) glycerol (all final concentrations) by heating at 10°C for 4 min. $NaDodSO_4$ gel electrophoresis of MC5b-9 in one-dimension under nonreducing conditions used a 5% acrylamide stacking and a 7% running gel. For two-dimensional separations, the second dimension was conducted under reducing conditions by incubating a gel section sliced from the first-dimension gel in 50 mM Tris/10 mM phosphate, pH 6.8/0.4% $NaDodSO_4$/2 mM EDTA/140 mM 2-mercaptoethanol for 40 min at 37°C. The gel slice was washed twice in 200 ml of this buffer containing 10 mM iodoacetamide but no 2-mercaptoethanol. The first-dimension gel slice was placed on top of a 7–12.5% exponential polyacrylamide gradient gel and immobilized by polymerization in 7% acrylamide stacking gel solution. After electrophoresis, gels were stained for protein with Coomassie brilliant blue R-250 as described (16). Autoradiography was conducted on dried gels with Kodak X-Omat AR film (Eastman). Molecular weights were estimated by utilizing the following reference proteins and indicated M_r: C5bα, 102,000; C5bβ, 75,000; C6, 124,000; C7, 104,000; C8α, 77,000; C8β, 70,000; C8γ, 20,000; and C9, 71,000. Densitometric scans (Quick Scan R&D spectrophotometer; Helena Laboratories, Beaumont, TX) of Coomassie blue-stained gels were utilized to estimate molar ratios of MC5b-9 subcomponents as described (29).

Antisera. Monospecific goat antisera to human C5 and C9 were prepared as described (24, 30). Caprine antisera to band X, as defined (16), were prepared in the following manner. Purified MC5b-9 was subjected to one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis under nonreducing conditions as outlined above. After electrophoresis, the slab gel was immersed in 4 M sodium acetate at 23°C for 60 min to visualize the protein bands without fixation or staining (31). The band corresponding to X protein was carefully excised, washed in 1 liter of water for 10 min, and stored at −70°C. In preparation for immunization, the gel slices were frozen in liquid nitrogen, powdered with a mortar and pestle, and emulsified in equal volumes of phosphate-buffered saline and Freund's complete adjuvant. Animals received multiple-site injections with approximately 200 μg of X protein every 4–6 weeks, and antisera were collected 7–10 days after each booster injection. The antisera were made 10 mM in EDTA and 0.02% in sodium azide and heat-inactivated at 56°C for 45 min. Ouchterlony double diffusion and one-dimensional rocket immunoelectrophoresis analyses were conducted as described (24).

Immunoreplication Procedure. Protein bands from one-dimensional $NaDodSO_4$ gels were electrophoretically transferred onto nitrocellulose (Schleicher & Schuell) by the method of Towbin *et al.* (32). The protein bands were visualized by staining the nitrocellulose blots with amido black (0.1%) or by incubating the unstained nitrocellulose blots in 10 mM Tris, pH 7.2/150 mM NaCl/2% bovine serum albumin (Tris-saline-albumin buffer) and a 1:10 dilution of the indicated antisera for 12 hr at 37°C. The antiserum-treated blots were incubated for 4 hr at 25°C in Tris-saline-albumin buffer containing 50 μg of ^{125}I-labeled protein A (Pharmacia, Uppsala, Sweden), radiolabeled with enzymobeads as described above to a specific activity of 5–8 × 10^5 cpm/μg. The nitrocellulose immunoreplicas were washed in 150 mM NaCl, dried, and exposed to Kodak X-Omat AR film for 1–4 days.

RESULTS

In an effort to characterize X protein further and to determine what relationship it might have to the other terminal C components, ^{125}I-radioiodinated highly purified C3, C5, C6, C7, C8, and C9 were individually incubated with normal human serum and EA at 37°C for 60 min. The resulting EAC membranes were washed and solubilized with zwitterionic detergent, and the 29S MC5b-9 dimeric complexes were isolated. The differentially labeled complexes and the ^{125}I-labeled C3b obtained from SB_{12}-solubilized EAC were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (nonreduced gels) and autoradiographic analysis. The results from these studies (Fig. 1) indicated that radiolabeled C5, C6, C7, and C8 incorporated into 29S MCb5-9 complexes expressed the anticipated subunit structure. However, 29S MC5b-9 complexes contained multiple C9 precursor-derived radiolabeled protein bands, one of which comigrated with X protein (upper arrow, Fig. 1, lane 7). Also evident was a C9 precursor-derived band with an electrophoretic mobility similar to that of C6 (M_r, 118,000) and a major C9 fragment having M_r 47,000 (lower arrow).

Additional experimental approaches were developed to substantiate this presumptive evidence that X protein was derived from precursor C9 upon MC5b-9 complex assembly. The X protein band was cut from nonfixed, nonstained $NaDodSO_4$ slab gels and used as immunogen to raise an anti-X protein antiserum in goats. The monospecificity of anti-X protein antiserum was evaluated over a wide range of antibody concentrations by rocket immunoelectrophoresis with normal human serum as described (24). The anti-X protein antiserum formed a precipitin line with only one component of normal human serum (Fig. 2*C*). Double-immunodiffusion analysis demonstrated that anti-X protein antiserum reacted with native monomeric C9 in normal human serum as evidenced by the formation of a precipitin line of complete identity with it and with a monospecific anti-C9 antiserum (Fig. 2*A*). Additional studies indicated that anti-X protein antiserum did not crossreact with C3, C5, C6, C7,

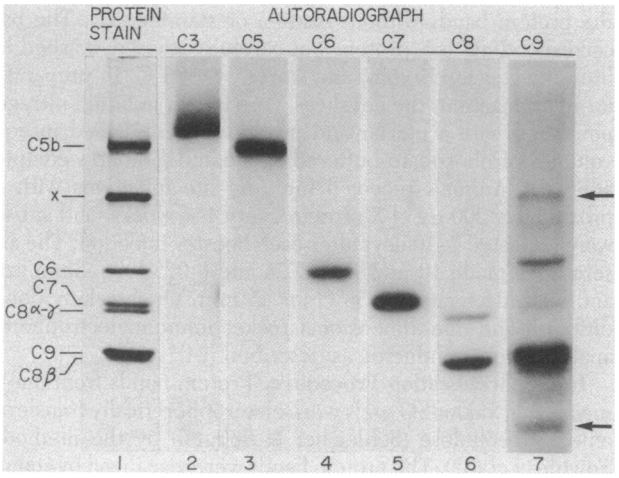

FIG. 1. NaDodSO$_4$/polyacrylamide gel (nonreduced) electrophoresis and autoradiography of purified 29S MC5b-9 complexes assembled in the presence of individual ^{125}I-radiolabeled C precursor proteins. Lane 1 shows Coomassie blue-staining pattern for 29S MC5b-9 complexes (82 μg); lanes 2–7 show autoradiograms. Lane 2, ^{125}I-C3b solubilized from EAC membranes with SB_{12} detergent. Lanes 3–7, isolated 29S MC5b-9 complexes assembled in the presence of various ^{125}I-labeled C components: 3, C5; 4, C6; 5, C7; 6, C8; 7, C9. Each lane contained approximately 3×10^3 cpm, and the autoradiogram was developed for 5 days.

or C8 when analyzed by double immunodiffusion with normal human serum and the corresponding monospecific antisera (data not shown). Anti-X protein antiserum was also reactive with fully assembled 29S MC5b-9 complexes (Fig. 2*B*).

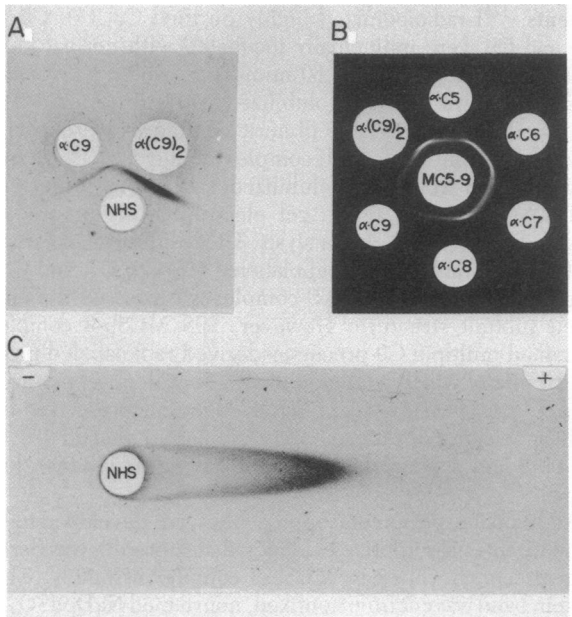

FIG. 2. Reactivity of caprine anti-X protein antiserum [$\alpha(C9)_2$]. (*A*) Double-immunodiffusion analysis; each well contained 20 μl of the indicated sample. Normal human serum (NHS) contained 10 mM EDTA. (*B*) Double-immunodiffusion analysis in which the center well contained 20 μl of purified 29S MC5b-9 complex (1 mg/ml) and the outer wells contained 20 μl of the indicated antisera. (*C*) Rocket immunoelectrophoresis of normal human serum plus 10 mM EDTA into an agarose slab (3.5 ml) containing 27 μl of anti-X protein antiserum per ml of agarose. Rocket immunoelectrophoresis was conducted over a wide range of antiserum concentrations in which up to 1.2 ml of anti-X protein antiserum was used per microscope slide (total volume of agarose slab, 3.5 ml) without visualization of contaminating antigen–antibody reactions.

The relationship between precursor C9 and X protein was further documented by immunoreplication procedures. The 29S MC5b-9 complex subcomponents were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis and transferred electrophoretically onto nitrocellulose blots. The nitrocellulose blots were stained for protein with amido black (Fig. 3, lane 2) or developed with various monospecific antisera followed by ^{125}I-radiolabeled protein A and autoradiography. The results obtained from this series of experiments demonstrated that anti-X protein antiserum reacted strongly with both monomeric C9 and X protein bands present in 29S MC5b-9 complexes (Fig. 3). Furthermore, monospecific antiserum raised to native human C9 also reacted with monomeric C9 and X protein bands present in 29S MC5b-9 complexes (Fig. 3, lane 4). Monospecific antisera to human C5, C6, C7, or C8 expressed no detectable reactivity with X protein under identical experimental conditions (data not shown).

Collectively, these data, in conjunction with the previously determined estimate of M_r 158,000 for X protein (16), indicate that the X protein present in isolated 29S MC5b-9 complex preparations represented a covalently bonded dimer of C9. This hypothesis was confirmed by two-dimensional NaDodSO$_4$ slab gel analysis of isolated 29S MC5b-9 complexes (Fig. 4). The first-dimension slab gel, which was run under nonreducing conditions, resolved the MC5b-9 complex subcomponents into a typical electrophoretic profile. The second dimension was run under reducing conditions in order to visualize the polypeptide chain subunit structure of each subcomponent. As indicated by the arrow in Fig. 4, the X protein ran in the second dimension as a single polypeptide chain exhibiting a M_r of 71,000 which comigrated with monomeric C9. The second-dimension slab gel also revealed the presence of additional 71,000 M_r C9 precursor-related protein bands. The results presented in Figs. 1 and 3 also support the conclusion that, during MC5b-9 complex assembly, monomeric C9 is able to interact covalently with other molecules to form various C9 "mixed" complexes in ad-

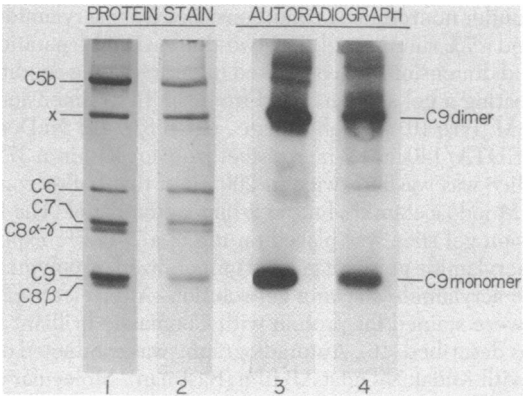

FIG. 3. Use of immunoreplication procedures for the detection of C9-related proteins present in the fully assembled 29S MC5b-9 complex. Purified 29S MC5b-9 complex (50 μg per lane) was separated by NaDodSO$_4$/polyacrylamide gel electrophoresis under nonreducing conditions. Lanes were cut from the gel and analyzed as follows: 1, Coomassie blue-staining pattern of NaDodSO$_4$ gel; 2, amido black protein staining pattern of 29S MC5b-9 complex transferred to nitrocellulose; 3, autoradiogram of 29S MC5b-9 complex nitrocellulose blot treated with anti-X protein antiserum and ^{125}I-labeled protein A; 4, same as lane 3 except the nitrocellulose blot was treated with anti-native C9 antiserum. The reactivity of the anti-native C9 antiserum with the NaDodSO$_4$ denatured protein blots obtained from NaDodSO$_4$ gels was greatly diminished and somewhat variable (lane 4) compared to that of the anti-X protein antiserum (lane 3) which was raised against the NaDodSO$_4$-denatured X protein cut from NaDodSO$_4$ gels as immunogen.

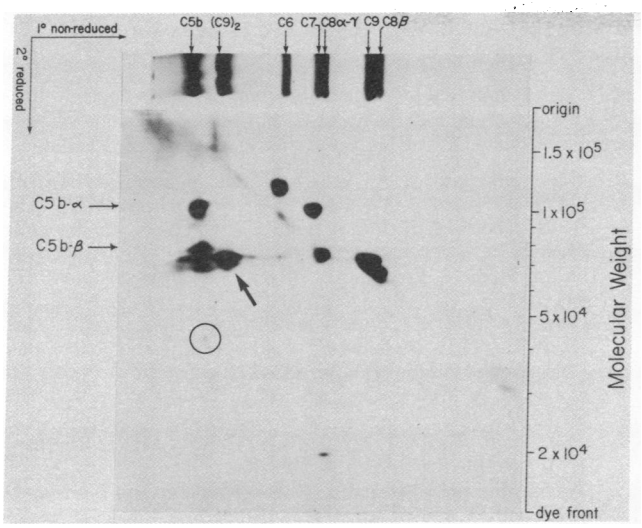

FIG. 4. Two-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis of 29S MC5b-9 complexes. First dimension (1°): 29S MC5b-9 complex (117 μg) was electrophoretically separated in the presence of $NaDodSO_4$ under nonreducing conditions on a 7% polyacrylamide slab gel; a parallel lane from this gel was stained with Coomassie blue and is shown above for orientation. Second dimension (2°): Electrophoresis was conducted in the presence of $NaDodSO_4$ under reducing conditions on a 7–12% exponential gradient polyacrylamide slab gel. Arrow, X protein.

dition to the C9 dimers. For example, the data presented are consistent with the following pattern of C9 covalent interactions: bonding of one C9 monomer with the 47,000 M_r C9 fragment to yield a 118,000 M_r complex with an electrophoretic mobility similar to that of C6; bonding of two C9 monomers to result in C9 dimer, which is the predominant disulfide-bonded form of C9 present in fully assembled 29S MC5b-9 complexes (Table 1); bonding of one C9 dimer with the 47,000 M_r C9 fragment to yield a 205,000 M_r complex comigrating with C5b (the presence of this 47,000 M_r C9 fragment is indicated by a circle in the second-dimension gel in Fig. 4); and bonding of three C9 monomers to result in C9 trimer formation to yield a complex migrating slightly above C5b (approximate M_r 220,000). Inclusion of free sulfhydryl reactive reagents—i.e., *p*-hydroxymercuribenzoate and iodoacetamide at final concentrations of 15 mM—during MC5b-9 complex extraction, isolation, and analysis on $NaDodSO_4$/polyacrylamide gel electrophoresis did not block or diminish the formation of C9 dimers or C9 mixed complexes.

Table 1. Subcomponent molar ratios of 29S MC5b-9 complex

Subcomponent	Molecular weight	Molar ratio
C5b	200,000	1.2 ± 0.3
$(C9)_2$	158,000	1.2 ± 0.4
C6	114,000	1
C7	95,000	1.3 ± 0.3
C8α-γ	93,000	0.78 ± 0.4
C8β	70,000	1.3 ± 0.5
C9	71,000	3.6 ± 0.7
C9 mixed complexes*	118,000–220,000	0.4

Molar ratios were estimated from densitometric scans of Coomassie blue-stained gels. Data are presented as mean ± SD for five individual 29S MC5b-9 preparations.

* Precursor-derived C9 complexes other than C9 dimers as defined in the text. The collective molar ratio for the C9 mixed-complex related protein was estimated from a horizontal scan of the 71,000 M_r protein bands as visualized in the second-dimension gel in Fig. 4 by defining the molar staining intensity of the C9 dimer as being equal to 1.0.

Molar ratio estimates of the subcomponents present in the 29S MC5b-9 complex preparations were made from densitometric scans of Coomassie blue-stained $NaDodSO_4$ gels as described (29). When the molar staining intensity for each subcomponent band was normalized to C6, the C5b, C9 dimer, C6, C7, C8α-γ, and C8β subcomponents were determined to be present in equimolar amounts with slightly more than 3 mol of monomeric C9 and 0.4 mol of C9 derived from mixed C9 complexes being present per mol of MC5b-9 complex (Table 1).

DISCUSSION

Several experimental approaches were used in these studies to identify the 158,000 M_r X protein present in fully assembled detergent-solubilized 29S MC5b-9 complexes as a disulfide-bonded C9 dimer: (*i*) radiolabeled monomeric C9 was incorporated into the X protein band upon complex assembly (Fig. 1, lane 7); (*ii*) antiserum raised to X protein reacted with MC5b-9 complexes (Fig. 2*B*) and formed a precipitin line of identity when analyzed by double immunodiffusion against normal human serum and anti-native C9 antiserum (Fig. 2*A*); (*iii*) immunoreplication procedures demonstrated that anti-X protein antiserum reacted with both monomeric C9 and X protein (Fig. 3, lane 3); (*iv*) monospecific anti-native C9 antiserum also reacted with monomeric C9 and X protein (Fig. 3, lane 4); and (*v*) two-dimensional $NaDodSO_4$/gel electrophoresis of 29S MC5b-9 complexes, with the second dimension run under reducing conditions, revealed that X protein was composed of two identical subunits that comigrated with monomeric C9 (Fig. 4). In addition, convincing evidence was also presented in Figs. 1, 3, and 4 to demonstrate that monomeric C9 can interact covalently with other C9 monomers, C9 fragments, and possibly other proteins to form various mixed complexes expressing C9 subunit structure and antigenic determinants. Furthermore, the assembly and formation of disulfide-bonded C9 complexes occurred during MC5b-9 complex assembly on target cell membranes and not during subsequent experimental manipulations because inclusion of *p*-hydroxymercuribenzoate and iodoacetamide during complex isolation and analysis had no effect on the formation of disulfide-bonded C9 complexes.

Molar ratio analyses of MC5b-9 subcomponents (Table 1) can reconcile conflicting reports concerning the subcomponent composition and M_r values assigned to fully assembled MC5b-9 complexes. By erroneously designating the 158,000 M_r X protein band as C5c, Bhakdi *et al.* (33) and Biesecker *et al.* (17) concluded that the C5b-9 monomeric complex solubilized from C-lysed membranes expressed an approximate M_r of 800,000 with molar ratios for C5b, C6, C7, C8, and C9 subcomponents of 1:1:1:1:2–4. However, upon identification of X protein as a C9 dimer, the correct molecular formula for the fully assembled MC5b-9 monomeric complex (Table 1) would be $(C5b, C6, C7, C8, C9_4)(C9)_2$. Translation of this formula into absolute molar ratios yields 1 mol of C5b, C6, C7, and C8 with 6 mol of C9 precursor-derived protein per MC5b-9 complex, with the total protein component having M_r 982,000 (see *Materials and Methods* for terminal component M_r designations). This conclusion is in close agreement with the molar ratios and calculated M_r analyses for fully assembled membrane-bound C5b-9 complex originally reported by Kolb *et al.* (7). In addition, our results are also in agreement with the recent studies by Bhakdi and Tranum-Jensen (18) who calculated the M_r contribution of the protein moiety contained within a monomeric MC5b-9 complex to be approximately 1×10^6.

One major distinction between MC5b-9 and SC5b-9 com-

plexes is the number of C9 molecules and the types of C9 interactions contained within each complex. As indicated in the present report, each MC5b-9 complex contains as many as 4 mol of monomeric C9 and 1 mol of C9 dimer whereas each SC5b-9 complex contains 3–4 mol of monomeric C9 and no C9 dimers (29). This comparison suggests that: (*i*) binding of S protein to nascent C5b67 complexes limits the number of C9 molecules that are able to bind per complex; (*ii*) a critical minimum of C9 molecules (three or four) must bind per complex before C9 dimers can be formed; and (*iii*) C9 dimer formation is closely associated with or is essential for MC5b-9 complex membranolytic function because SC5b-9 complexes are hemolytically inactive and do not exhibit ring-like ultrastructure as visualized by electron microscopy (17). These conclusions may be partially compatible with the proposals of Boyle *et al.* (21, 34) who postulated that the ratio between C9 and C8 molecules was critical for MC5b-9 complex functional expression. It appears from our studies that saturation of all C9 binding sites during complex assembly—i.e., the stepwise increase in the C9/C8 ratio to a maxium of 6—is required for C9 dimer formation and expression of lytic activity. However, it is becoming increasingly clear from data now available (16, 17, 19, 20, 22, 35) that the observed heterogeneity in the functional pore size of C lesions is not dependent upon C9/C8 ratios but rather is a function of the total number and aggregation state of fully assembled C5b-9 complexes residing on the target cell membrane. The higher the MC5b-9 aggregation state, the larger the functional lesion (see ref. 22 for detailed discussion of this hypothesis).

In view of the currently prevailing theory that the major configuration of C-attack-complexes on target cell membrane surfaces is a MC5b-9 dimer (16, 17, 19, 22) that represents the fundamental ultrastructural and functional unit which mediates C-directed target cell membranolysis (17, 19, 22), it is tempting to speculate that the C9 dimers present in the 29S MC5b-9 complex dimers form intercomplex, covalently bonded, disulfide crosslinks (Fig. 5*A*). This process would provide a mechanism to stabilize the MC5b-9 dimeric aggregation state and possibly provide a mechanism to stabilize higher-ordered MC5b-9 aggregation states resulting in the ordered enlargement of functional lesions on target cell membrane surfaces under C attack. However, in the absence of detailed characterizations of stable MC5b-9 complex monomers in free solution and a more complete understanding of the biochemical characteristics of the precursor C9 molecule, the possibility that C9 dimers represent intracomplex stabilizing, covalent crosslinks cannot be dismissed (Fig. 5*B*).

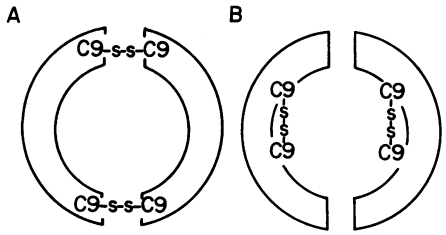

FIG. 5. Possible arrangements of C9 dimers in relationship to the structure of 29S MC5b-9 complex dimers. (*A*) Crosslinking of two MC5b-9 complexes by two intercomplex, disulfide-bonded C9 dimers. (*B*) Two MC5b-9 complexes held together by noncovalent interactions and containing two intracomplex, disulfide-bonded C9 dimers. Each model provides that 1 mol of C9 dimer is present per mol of MC5b-9 complex.

C.F.W. is supported by National Institute of Allergy and Infectious Disease Postdoctoral Fellowship AI-06000. This investigation was supported by Grant ROICA 24447 from the National Cancer Institute.

1. Müller-Eberhard, H. J. (1975) *Annu. Rev. Biochem.* **44**, 697–724.
2. Müller-Eberhard, H. J. (1969) *Annu. Rev. Biochem.* **38**, 389–414.
3. Müller-Eberhard, H. J. & Schreiber, R. D. (1980) *Adv. Immunol.* **29**, 1–53.
4. Porter, R. R. & Reid, K. B. M. (1978) *Nature (London)* **275**, 699–703.
5. Götze, O. (1975) in *Proteases and Biological Control*, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 255–272.
6. Götze, O. & Müller-Eberhard, H. J. (1970) *J. Exp. Med.* **132**, 898–915.
7. Kolb, W. P., Haxby, J. A., Arroyave, C. M. & Müller-Eberhard, H. J. (1972) *J. Exp. Med.* **135**, 549–566.
8. Tranum-Jensen, J., Bhakdi, S., Bhakdi-Lehnen, B., Bjerrum, O. J. & Speth, V. (1978) *Scand. J. Immunol.* **7**, 45–56.
9. Esser, A. F., Kolb, W. P., Podack, E. R. & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1410–1414.
10. Hugli, T. E. (1978) *Adv. Immunol.* **26**, 1–53.
11. Podack, E. R., Biesecker, G., Kolb, W. P. & Müller-Eberhard, H. J. (1978) *J. Immunol.* **121**, 484–490.
12. Podack, E. R., Biesecker, G. & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 897–901.
13. Hammer, C. H., Shin, M. L., Abramovitz, A. S. & Mayer, M. M. (1977) *J. Immunol.* **119**, 1–26.
14. Shin, M. L., Paznekos, W. A., Abramovitz, A. S. & Mayer, M. M. (1977) *J. Immunol.* **119**, 1358–1364.
15. Bhakdi, S., Bjerrum, O. J., Rother, U., Knufermann, H. & Wallach, D. F. H. (1975) *Biochem. Biophys. Acta* **406**, 21–35.
16. Ware, C. F., Wetsel, R. A. & Kolb, W. P. (1981) *Mol. Immunol.* **18**, 521–531.
17. Biesecker, G., Podack, E. R., Halverson, C. A. & Müller-Eberhard, H. J. (1979) *J. Exp. Med.* **149**, 448–458.
18. Bhakdi, S. & Tranum-Jensen, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1818–1822.
19. Podack, E. R. & Müller-Eberhard, H. J. (1981) *J. Biol. Chem.* **256**, 3145–3148.
20. Sims, P. J. & Lauf, P. K. (1980) *J. Immunol.* **125**, 2617–2625.
21. Boyle, M. D. P., Gee, A. P. & Borsos, T. (1979) *J. Immunol.* **123**, 77–82.
22. Esser, A. F. (1981) in *Biological Membranes*, ed. Chapman, D. (Academic, New York), Vol. 4, in press.
23. Tack, B. F. & Prahl, J. W. (1976) *Biochemistry* **15**, 4513–4521.
24. Wetsel, R. A., Jones, M. A. & Kolb, W. P. (1980) *J. Immunol. Methods.* **35**, 319–335.
25. Podack, E. R., Kolb, W. P., Esser, A. F. & Müller-Eberhard, H. J. (1979) *J. Immunol.* **123**, 1071–1077.
26. Kolb, W. P. & Müller-Eberhard, H. J. (1976) *J. Exp. Med.* **143**, 1131–1139.
27. Hadding, U. & Müller-Eberhard, H. J. (1969) *Immunology* **16**, 719–735.
28. Laemmli, V. K. (1970) *Nature (London)* **227**, 680–685.
29. Kolb, W. P. & Müller-Eberhard, H. J. (1975) *J. Exp. Med.* **141**, 724–735.
30. Kolb, W. P. & Müller-Eberhard, H. J. (1974) *J. Immunol.* **113**, 479–488.
31. Higgins, R. C. & Dahmus, M. E. (1979) *Anal. Biochem.* **93**, 257–260.
32. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
33. Bhakdi, S., Ey, P. & Bhakdi-Lehnen, B. (1976) *Biochim. Biophys. Acta.* **419**, 445–457.
34. DeLisi, C., Boyle, M. & Borsos, T. (1980) *J. Immunol.* **125**, 2055–2062.
35. Sims, P. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1838–1842.