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Biochemical Characterization of the Sixth Component (C6) of Human Complement†

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ABSTRACT: The sixth component (C6) of complement was purified from human serum in fully hemolytically active form by anti-C6 and anti-impurities immunoadsorbent column chromatography. When 3-4 L of pooled normal human serum containing 10 mM EDTA as starting material was employed, the final C6 preparations exhibited yields ranging from 40% to 56%, with 1780-1940-fold purification factors based on recovery of specific hemolytic activity. Highly purified C6 was found to be a relatively stable serum glycoprotein, containing 11.3% carbohydrate, that retained 80-100% functional hemolytic activity upon incubation under the following conditions: (1) 6 M guanidine hydrochloride or 6 M urea at 37 °C for 3 h, (2) 4 M potassium thiocyanate at 4 °C for 18 h, (3) 56 °C for 90 min, or (4) pH 5-11 at 37 °C for 2 h. C6 exhibited 4.7 *p*-(chloromercuri)benzoate (pCMB) and 6.3 DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] binding sites per molecule; the C6 hemolytic activity was completely inhibited by 6 mM mercuric chloride and 10 mM pCMB. C6 was highly susceptible to inactivation by trypsin or thermolysin, with a 50% reduction in hemolytic activity occurring after 30 min at 37 °C with 0.2% and 0.4% w/w, respectively. The C6 functional activity was also inhibited by diisopropyl fluorophosphate (DIFP), *p*-tosyl-L-lysyl chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), and dansyl fluoride over a concentration range of 10^{-2}–10^{-3} M; (*p*-amidinophenyl)methanesulfonyl fluoride (pAPMSF) was inhibitory over a concentration range of (0.5–2) × 10^{-4} M. The synthetic substrate acetylglycyl-L-lysine methyl ester was able to completely protect C6 from inactivation by pAPMSF but was unable to protect C6 from inactivation by mercuric chloride or pCMB. These results strongly suggest that C6 is a serine active site protease and that expression of the C6 enzymatic activity is essential for complement membrane attack complex (MC5b-9) membranolytic function.

Complement (C) is a sequential, multimolecular system of plasma proteins which can be activated by a variety of immunological as well as nonimmunological stimuli (Müller-Eberhard, 1975). C activation, which can proceed via either the classical or alternative pathway, is mediated through a series of cascading reaction steps which are dependent upon the conversion of serum zymogens to active serine esterase enzymes. The classical C pathway is activated by IgG and IgM containing immune complexes and is composed of 11 plasma proteins which are identified numerically as C1–C9 (Müller-Eberhard, 1969). The first component (C1) is a calcium-dependent complex of three plasma proteins, C1q, C1r, and C1s (Lepow et al., 1963). Activated C1r, C1s, and C2 represent the three proteolytic enzymes of the classical pathway which have been described previously (Haines & Lepow, 1964; Naff & Ratnoff, 1968; Porter & Reid, 1978). The alternative C pathway is activated by plant, fungal, and bacterial polysaccharides and lipopolysaccharides in particulate form and is composed of 11 plasma proteins identified as factor D, factor B, properdin, the modulating proteins β_1H and C3b INA, and C3–C9 (Müller-Eberhard & Schreiber, 1980). The C3–C9 components are thus common to both C pathways. Factor D, C3b INA, and activated factor B represent the three proteolytic enzymes of the alternative C pathway which have been described previously (Müller-Eberhard & Götze, 1972; Götze, 1975; Pangburn et al., 1977).

Activation of either C pathway results in the expression of multiple biological activities which include (1) deposition of C3b molecules on the surface under C attack, resulting in particle opsonization and clearance by C3 receptor positive neutrophils, monocytes, and macrophages (Lay & Nussenz-

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‡ Recipient of Research Career Development Award CA-00412 from the National Cancer Institute, Department of Health, Education and Welfare.

0006-2960/82/0421-0294$01.25/0

weig, 1968; Huber et al., 1968), (2) production and release of C3a and C5a anaphylatoxins (Hugli, 1978), and (3) assembly of the C5b-9 membrane attack complex which mediates the irreversible damage to biological membranes associated with C-dependent cytolysis (Götze & Müller-Eberhard, 1970; Kolb et al., 1972).

Although the molecular weights and subunit structures of the individual precursor proteins of the C5b-9 complex have been reported previously (Tack et al., 1979; Podack et al., 1976; Kolb & Müller-Eberhard, 1975; Hadding & Müller-Eberhard, 1969), very little biochemical information is available describing these proteins. We therefore wish to report our results on the initial biochemical characterization of human C6.

Experimental Procedures

Materials. Outdated human plasma containing 10 mM citrate as an anticoagulant was obtained from the blood bank at the Bexar County Hospital, The University of Texas Health Science Center, San Antonio, TX. Serum was obtained by clotting outdated plasma upon addition of $CaCl_2$ to a final concentration of 20 mM for 2 h at 37 °C. Serum was also prepared from freshly clotted whole blood, 1–2 h at 37 °C, obtained from consenting healthy human volunteers. Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; EDTA,[1] guanidine hydrochloride (Gdn·HCl), urea, sodium phosphate, acetonitrile, and phenol reagent were purchased from Fisher Scientific Co., Fair Lawn, NJ. TLCK, TPCK, pCMB, DIFP, and sodium barbital were obtained from Sigma Chemical Co., St. Louis, MO; CNBr, 2-mercaptoethanol, dithiothreitol, 2-iodoacetamide, 2-iodoacetate, PMSF, and Coomassie Brilliant Blue R-250 were purchased from Eastman Kodak Co., Rochester, NY. EACA was purchased from Schwarz/Mann, Orangeburg, NY; *N*-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich Co., Milwaukee, WI. $HgCl_2$ was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ; agarose, electrophoresis grade acrylamide, *N*,*N*′-methylenebis(acrylamide), TEMED, and ammonium persulfate were purchased from Bio-Rad Laboratories, Richmond, CA; 1 × crystallized bovine pancreatic, TPCK-treated trypsin, and 3 × crystallized thermolysin were purchased from Calbiochem, San Diego, CA. pAPMSF was the generous gift of Dr. David H. Bing, Center for Blood Research, Boston, MA.

Antisera Production and IgG Purification. Antisera were raised to human C6, evaluated, and rendered monospecific by solid-phase adsorption procedures described previously for human C5 (Wetsel et al., 1980). The C6 antigen utilized for anti-C6 antisera production was prepared as described by Podack et al. (1976). Antisera to contaminant proteins present in the C6 preparations (anti-impurities antisera) were prepared in goats as indicated above by injecting the C6 depleted, nonadsorbed protein fractions obtained upon passage of the starting material (a resuspended 0–40% ammonium sulfate precipitate of NHS) through the anti-C6 immunoadsorbent column. The IgG fraction was prepared from 600 mL of monospecific anti-human C6 or 400 mL of anti-impurities antisera by standard procedures (Fudenberg, 1967).

Solid-Phase Immunoadsorbent Preparation. The purified IgG fraction, obtained from either goat anti-C6 or anti-impurities antisera, was dialyzed extensively against coupling buffer (16 g of $NaCHO_3$, 1.1 g of Na_2CO_3, and 58.4 g of NaCl per 2 L, pH 9.0) and covalently coupled to CNBr-activated Sepharose CL-4B at 30 mg of IgG/mL of beads by a modification of the procedure of March et al. (1974) as described by Kolb et al. (1979).

C6 Hemolytic Assay. Immunochemically, C6-depleted serum (C6D) was prepared by passage of freshly drawn human serum (200 mL) containing 10 mM EDTA through an anti-C6–Sepharose CL-4B column (5 × 12 cm). The C6D breakthrough fractions were pooled and employed in the C6 hemolytic assay which contained the following: (a) GVB plus the sample to be assayed in a final volume of 280 μL, (b) 20 μL of C6D reconstituted with 80 μg of C1q/mL and 20 μL of a 0.3 M $CaCl_2$ and 1 M $MgCl_2$ stock/mL, and (c) 200 μL of EA at a concentration of 1.5×10^8/mL. The assay mixtures were incubated at 37 °C with frequent mixing for 30–60 min, and the reaction was stopped by transferring the tubes to an ice bath and adding 1 mL of ice-cold GVB. The unlysed cells were removed by centrifugation, and the extent of hemolysis was determined spectrophotometrically at 412 nm. One effective molecule was defined as the amount of C6 required to lyse one EA. Experimentally this was the quantity of C6 required to lyse 63.3% of a given number of EA, usually 3×10^7 EA/assay.

Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of $NaDodSO_4$, was conducted as described by Laemmli (1970). Subsequent to electrophoresis, the gels were fixed in 10% Cl_3CCOOH–20% methanol, stained in 25% methanol–10% acetic acid containing 0.25% Coomassie Blue, and destained in 10% methanol–10% acetic acid.

Amino Acid and Carbohydrate Analyses. Analysis of amino acids was performed with a Durrum Model D-500 automatic amino acid analyzer equipped with a computerized integrator. Triplicate samples were hydrolyzed for 24, 48, or 72 h with 6 N HCl at 110 °C in evacuated and sealed tubes. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation. Sialic acid was determined by the procedure of Aminoff (1961). Hexose sugars were determined by the anthrone procedure of Loewus (1952) after hydrolysis at 100 °C in 2 N HCl for 4 h employing glucose as a standard. Hexosamines were determined by the procedure of Pesez & Bartos (1974) after hydrolysis at 100 °C in 3 N HCl for 4 h employing galactosamine as a standard. Total reducing sugars were determined by the procedure of Park & Johnson (1949) after hydrolysis at 100 °C in 3 N HCl for 4 h employing glucose as a standard. Many different hydrolysis conditions were investigated for the various carbohydrate assays, and the conditions reported above resulted in the maximum release of carbohydrate for each assay.

Protein Determinations. Protein determinations were conducted by the procedure of Lowry et al. (1951). In the case of highly purified C6, an $E^{1\%}_{1cm}$ at 280 nm of 10.77 was employed, which was determined by the microbiuret procedure as described by Bailey (1967) employing bovine serum albumin as a standard.

Results

Purification of Human C6. Effective utilization of immu-

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; TLCK, *p*-tosyl-L-lysyl chloromethyl ketone; TPCK, 1-(tosylamido)-2-phenylethyl chloromethyl ketone; pCMB, *p*-(chloromercuri)benzoate; DIFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; pAPMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; DTT, dithiothreitol, 2-ME, 2-mercaptoethanol; SAS, saturated ammonium sulfate; NEM, *N*-ethylmaleimide; IAA, 2-iodoacetamide; IOAc, 2-iodoacetate; $NaDodSO_4$, sodium dodecyl sulfate; EACA, ε-aminocaproic acid; TEMED, *N*,*N*,*N*′,*N*′-tetramethylenediamine; NHS, normal human serum; PBS, phosphate-buffered saline containing 5 mM phosphate and 145 mM NaCl, pH 7.4; PBS-E, PBS containing 10 mM EDTA; VB, isotonic veronal buffered saline, pH 7.4, containing 5 mM barbital, 145 mM NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$; GVB, VB containing 0.1% gelatin; EA, antibody-sensitized sheep erythrocytes.

Table I: Purification of C6[a]

fraction	vol (mL)	act. (units/mL × 10^{-4})[b]	total act. (units × 10^{-7})	yield (%)	protein (mg/mL)	sp act. (units/mg)	purification
human serum	3000	3.13	9.38	100	73.1	427	1
ammonium sulfate (0–40%)	1545	4.05	6.26	66.7	38.2	1060	2.48
anti-C6 immunoadsorbent	780	6.76	5.27	56.2	0.48	144000	330
anti-impurities immunoadsorbent	6.2	731.40	4.53	48.2	9.01	812000	1902

[a] Values based on C6 preparation 21. [b] One unit corresponds to 3 × 10^7 effective molecules; incubation time 40 min at 37 °C.

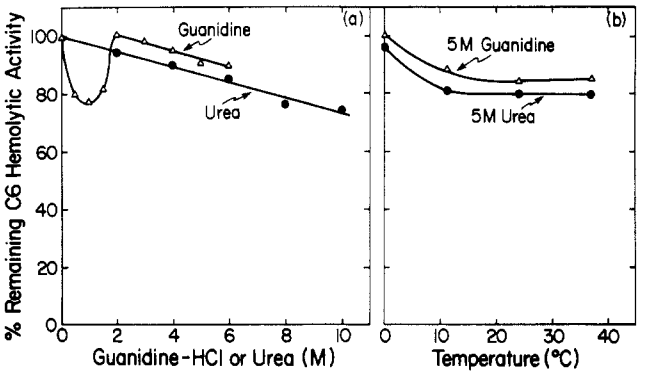

FIGURE 1: Stability of human C6 upon incubation with Gdn·HCl or urea. (a) Highly purified C6, 100 μg, was incubated with the indicated final concentrations of Gdn·HCl or urea in a total volume of 0.1 mL at 37 °C for 3 h. A 10-μL portion of each sample was diluted in 50 mL of ice-cold GVB, and 25-μL portions of each dilution were assayed in duplicate for remaining C6 hemolytic activity as described under Experimental Procedures. (b) C6, 100 μg, was incubated with a final concentration of either 5 M Gdn·HCl or 5 M urea in a total volume of 0.1 mL at the indicated temperatures for 3 h and assayed for remaining C6 hemolytic activity as described above.

noadsorbent affinity chromatographic procedures for the isolation of protein antigens requires the establishment of appropriate conditions for the dissociation of antigen–antibody complexes with retention of functional activity of the protein under investigation. Therefore, the stability of highly purified C6 upon incubation with increasing concentrations of protein denaturants frequently employed for the dissociation of antigen–antibody complexes was investigated. As indicated in panel a of Figure 1, C6 hemolytic activity gradually decreased upon incubations with increasing concentrations of Gdn·HCl or urea at 37 °C for 3 h. However, full C6 hemolytic activity was retained upon incubation with 5 M Gdn·HCl for 3 h if the temperature was lowered to 0–2 °C (Figure 1, panel b). Therefore, C6 purification was conducted at 1–4 °C, and 5 M Gdn·HCl was employed to elute C6 from the anti-C6 immunoadsorbent column (Figure 2).

The C6 containing protein fraction was obtaned from 3 L of outdated NHS by precipitation with ammonium sulfate at 40% saturation and 2 °C. The precipitate was redissolved to 3 L of PBS and fractionated again as indicated above. The C6-containing precipitate was finally dissolved in 1.5 L of PBS-E and applied to the anti-C6 immunoadsorbent column at a flow rate of 80 mL/h collecting 14-mL fractions (Figure 2, panel a). The immunoadsorbent column was washed with 3 L of PBS-E containing 2 M NaCl and 1% Triton X-100 (TX-100) to remove nonspecifically bound protein and 1 L of PBS-E containing 2 M NaCl to remove TX-100. Antibody-bound C6 was dissociated and eluted with 0.5 L of 5 M Gdn·HCl, and the C6-containing fractions were pooled immediately as indicated and exhaustively dialyzed against PBS. The anti-C6 immunoadsorbent column was requilibrated immediately with PBS-E which resulted in full restoration of the

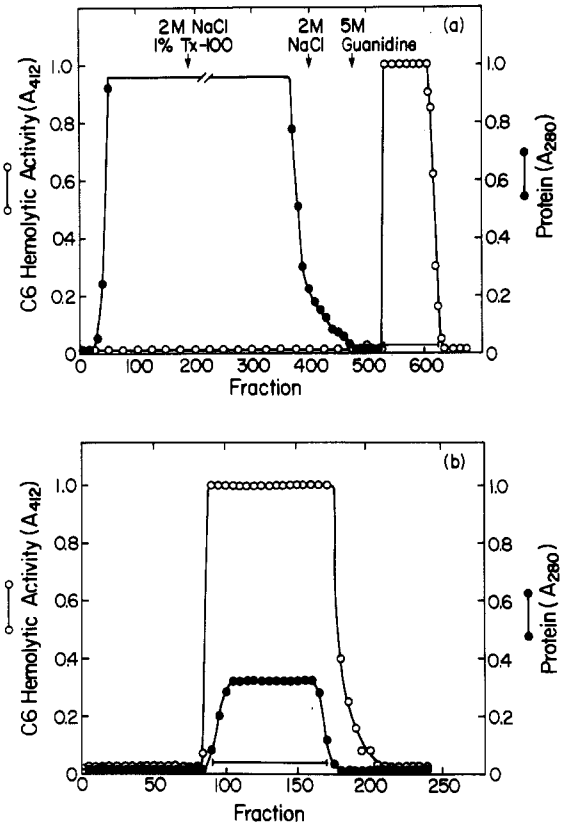

FIGURE 2: C6 isolation. (a) The solubilized ammonium sulfate fraction (0–40%) obtained from 3 L of NHS was applied to an anti-C6 immunoadsorbent column (6.5 × 18 cm) at 4 °C as described in the text. C6 was eluted with 0.5 L of 5 M Gdn·HCl at a flow rate of 100 mL/h collecting 5-mL fractions. (b) The dialyzed C6 pool obtained from the anti-C6 immunoadsorbent column was applied to an anti-impurities immunoadsorbent column (5 × 20 cm) at a flow rate of 50 mL/h collecting 5-mL fractions.

antibody binding capacity. The same immunoadsorbent column has been employed for more than 25 C6 preparations over the past 24 months without appreciable loss of C6 binding capacity. After dialysis, the C6-containing pool was applied to an anti-C6 impurities immunoadsorbent column (Figure 2, panel b). The C6-containing fractions were pooled as indicated, concentrated by positive pressure Amicon ultrafiltration, and stored at −70 °C. The anti-C6 impurities immunoadsorbent column was regenerated by application of 0.5 L of 3 M Gdn·HCl followed by 2 L of PBS at a flow rate of 200 mL/h. The isolation procedure is summarized in Table I. The final yield of C6 hemolytic activity ranged between 40% and 56% recoveries with purification values of 1780–1940-fold.

$NaDodSO_4$–Polyacrylamide Gel Electrophoretic and Immunochemical Analyses. Figure 3 shows the $NaDodSO_4$–polyacrylamide slab gel analysis of the individual C6 purification steps. As seen in lane 2, the ammonium sulfate fractionation step removed the albumin as well as other serum

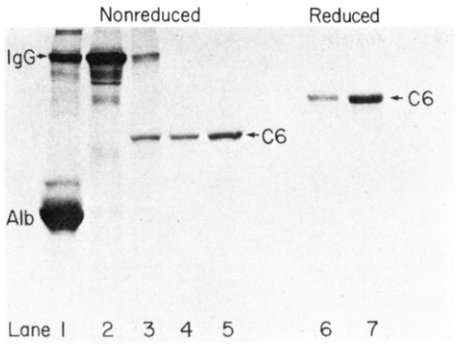

FIGURE 3: $NaDodSO_4$–polyacrylamide slab gel analysis. Samples in lanes 1–5 were run under nonreducing conditions, and samples in lanes 6 and 7 were run in the presence of 2-ME on a 7.5% polyacrylamide slab gel as outlined under Experimental Procedures. (Lane 1) NHS (40 μg); (lane 2) 40 μg of the C6-containing pool after ammonium sulfate fractionation of NHS; (lane 3) 40 μg of the C6-containing pool after anti-C6 immunoadsorbent chromatography; (lanes 4 and 5) 10 and 25 μg of the final C6 preparation obtained after anti-impurities immunoadsorbent chromatography; (lanes 6 and 7) same as 4 and 5 except samples were reduced with 1% 2-ME.

proteins from the C6-containing pool. The anti-C6 immunoadsorbent column step removed the majority of the contaminating serum proteins (lane 3), while the anti-impurities immunoadsorbent column removed the few remaining protein contaminants, predominantly IgG, yielding highly purified C6 (lanes 4–7).

Ouchterlony analysis of C6 preparations (1 mg/mL) revealed a precipitin line with anti-human C6, but no precipitin lines were evident when tested against anti-IgM, -IgG, -C7, -NHS, or -IgA (Figure 4, panel A). Ouchterlony patterns obtained with anti-human C6 showed a reaction of antigenic identity between the C6 present in NHS and C6 purified by immunoadsorbent chromatography (Figure 4, panel B), indicating that elution of C6 with 5 M Gdn·HCl had no demonstrable effect on C6 antigenic expression. Furthermore, all detectable C6 antigenic determinants were removed from NHS after passage through the anti-C6 immunoadsorbent column during the preparation of C6D (Figure 4, panel C).

Biochemical Characterization. (*a*) *General Properties.* As indicated in Table II, highly purified C6 was stable to heating at 56 °C for 90 min in GVB and preincubation with 200 mM EDTA, 1 M EACA, and 4 M KSCN or pH 5–11. The C6 hemolytic activity in NHS was surprisingly more susceptible to inactivation at 56 °C ($T_{1/2}$ = 20 min) than in purified form ($T_{1/2}$ > 4 h). Purified C6 was susceptible to inactivation by the reducing agents DTT and 2-ME. The presence of carbohydrates, especially sialic acid, that was important for hemolytic activity was suggested by the inactivation of C6 with (1–5) × 10^{-3} M sodium periodate.

Table II: Stability of Human C6 to Various Treatments[a]

treatment	incubation conditions	50% inactivation[b]
56 °C	purified C6, 40 μg/mL GVB	*c*
56 °C	NHS	20 min[d]
dithiothreitol	VB,[e] pH 7.4, 37 °C, 1 h	0.3 mM
2-mercaptoethanol	VB, pH 7.4, 37 °C, 1 h	14 mM
EDTA, 5–200 mM	VB, pH 7.4, 37 °C, 3 h	no effect
ε-aminocaproic acid, 0.1–1 M	VB, pH 7.4, 37 °C, 4 h	no effect
KSCN, 0.1–4 M	VB, pH 7.4, 4 °C, 18 h	no effect
sodium periodate	VB, pH 7.4, 37 °C, 1 h	4 mM
pH 2–12	37 °C, 1 h	*f*

[a] Highly purified C6 was employed at 0.1 mg/mL unless stated otherwise. [b] Determined by C6 hemolytic assay. [c] Activity of 80–90% remaining after 90 min, 56 °C. [d] Activity of 10% remaining after 90 min, 56 °C. [e] Veronal buffered saline. [f] No effect over pH range 5–11, 60–90% activity remaining between pH 2 and 3.5, and 20% remaining at pH 12.

(*b*) *Amino Acid and Carbohydrate Analyses.* Amino acid and carbohydrate analyses (Table III) were conducted as outlined under Experimental Procedures and indicated that C6 is a serum glycoprotein containing 11.3% carbohydrate, assuming a molecular weight of 125 000 (Podack et al., 1979). The relatively high content of half-cystine amino acid residues (6 mol %) in the C6 molecule suggests the presence of extensive intramolecular disulfide bonding which may account for the preservation of C6 functional activity after incubation with protein denaturants or heating at 56 °C.

(*c*) *Free Sulfhydryls.* Highly purified C6, at a final concentration of 50 μg/mL (4 × 10^{-7} M), was incubated with increasing concentrations of IAA, IOAc, DTNB, and pCMB at pH 8.2, HgCl at pH 7.4, and *N*-ethylmaleimide (NEM) at pH 7.0 for 3 h at 37 °C. As indicated in panel a of Figure 5, C6 was inactivated by HgCl and pCMB at concentrations of less than 10^{-2} M, while significant inactivation with DTNB, IAA, IOAc, or NEM required concentrations in excess of 10^{-2} M. Several experiments were conducted by employing [^{14}C]pCMB (10^{-2}–10^{-3} M) that demonstrated the presence of 4.7 ± 0.4 (mean ± SD, n = 3) pCMB binding sites per C6 molecule (Figure 5, panel b). Even though greater than 90% of the [^{14}C]pCMB was removed upon dialysis against 1 mM cysteine, the C6 hemolytic activity was not restored. The results of the pCMB binding studies were confirmed by employing DTNB (Ellman's reagent) by incubation of highly purified C6 (2 mg/reaction mixture) at 37 °C for 4 h with

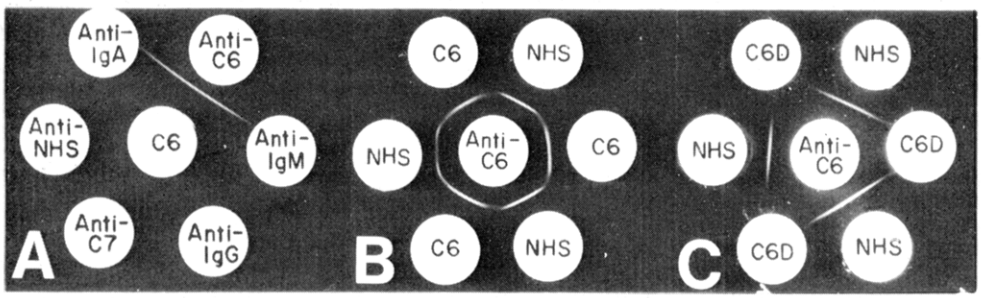

FIGURE 4: Ouchterlony analyses. (a) A 20-μL sample of highly purified C6 (1 mg/mL) was placed in the center well, and 10-μL samples of anti-C6, -IgM, -IgG, -C7, -NHS, and -IgA were placed in the outer wells as indicated. (b) A 20-μL sample of a 1:16 dilution of anti-C6 was placed in the center well, and a 20-μL sample of undiluted NHS–10 mM EDTA or C6 (65 μg/mL) was placed in the outer wells as indicated. (c) A 20-μL sample of a 1:16 dilution of anti-C6 was placed in the center well, and a 20-μL sample of undiluted NHS–10 mM EDTA or C6D–10 mM EDTA was placed in the outer wells as indicated.

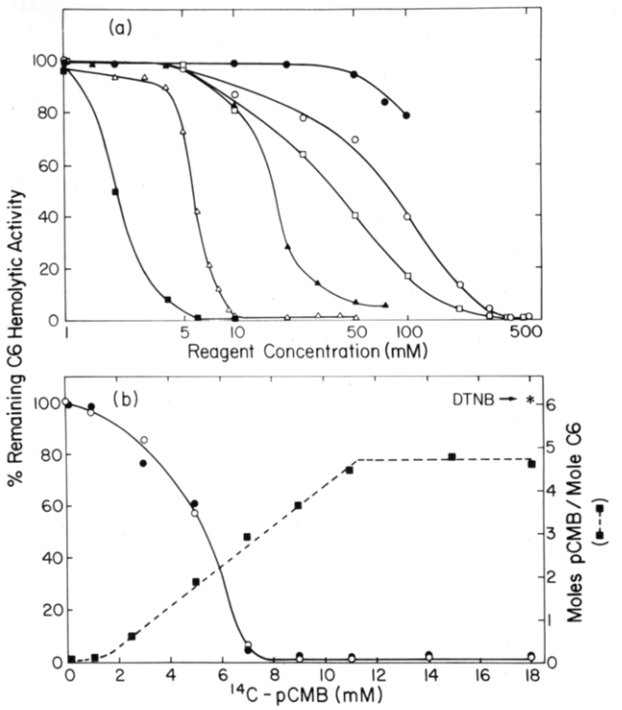

FIGURE 5: Free sulfhydryls. (a) C6 (4×10^{-7} M) was incubated at 37 °C with the indicated concentrations of the following reagents and assayed for remaining C6 hemolytic activity as outlined in the text: HgCl (■), pCMB (△), DTNB (▲), iodoacetate (□), iodoacetamide (○), *N*-ethylmaleimide (●). (b) One milligram of C6 was incubated in 5 mM Tris and 100 mM NaCl, pH 8.2, with the indicated concentrations of [^{14}C]pCMB (8.94×10^5 cpm/μmol) in a final volume of 400 μL at 37 °C for 2 h (final C6 concentration was 2×10^{-5} M). One-hundred microliters of each sample was exhaustively dialyzed against PBS or PBS containing 1 mM cysteine. The remaining hemolytic activity, [^{14}C]pCMB bound, and absorbance at 280 nm were determined for each dialyzed C6 sample. The [^{14}C]pCMB–CPM bound to each sample dialyzed against cysteine was subtracted from the [^{14}C]pCMB–CPM bound to the corresponding sample dialyzed vs. PBS and the pCMB binding sites per C6 molecule were determined. C6 hemolytic activity before dialysis (○); C6 hemolytic activity after dialysis against 1 mM cysteine (●).

100 mM DTNB in the presence or absence of 2% $NaDodSO_4$ in a final volume of 1 mL of 0.15 M Tris-HCl and 0.1 mM EDTA buffer, pH 8.2. The samples were exhaustively dialyzed vs. PBS-E, pH 8.2, and the DTNB specifically bound per C6 molecule was determined spectrophotometrically at 412 nm upon the addition of DTT to a final concentration of 20 mM (6.3 mol of DTNB/mol of C6 with or without the addition of $NaDodSO_4$; Figure 5, panel b).

(*d*) *Inactivation by Proteolytic Enzymes.* Highly purified C6, at a final concentration of 1 mg of C6/mL, was incubated with increasing amounts of thermolysin or TPCK–trypsin at 37 °C for 30 min. As indicated in Figure 6, human C6 was very susceptible to trypsin digestion, with concomitant loss of hemolytic activity. The molecular weights of the trypsin produced C6 fragments (Figure 6, insert) were determined to be the following: C6 fragment 1, M_r 103 000; 2, M_r 72 000; 3, M_r 43 500; 4, M_r 38 500; 5, M_r 31 000; 6, M_r 25 000.

(*e*) *Inactivation by Serine Protease Inhibitors.* Highly purified C6, at a final concentration of 50 μg/mL (4×10^{-7} M), was incubated with increasing concentrations of DIFP, PMSF, TLCK, TPCK, dansyl fluoride, and soybean trypsin inhibitor (SBTI) at 37 °C for 3 h in VB, pH 7.4. As seen in Figure 7, DIFP, PMSF, and TLCK inhibited C6 hemolytic activity over a concentration range of $10^{-2} - 10^{-3}$ M. Dansyl fluoride was not as effective, and SBTI had no effect. The results obtained with TPCK were inconclusive since TPCK induced C6 precipitation. In all protease inhibitor experiments,

Table III: Amino Acid and Carbohydrate Compositions of Human C6

	residues/100 residues	
	this paper[a]	Podack et al. (1979)
amino acid		
aspartic acid	10.04	9.83
threonine[b]	5.50	5.70
serine[b]	8.52	8.51
glutamic acid	12.82	12.36
proline	4.45	6.05
glycine	8.11	7.98
alanine	4.45	5.17
half-cystine[c]	6.25	4.86
valine	5.19	6.02
methionine[c]	0.50	0.90
isoleucine[d]	3.64	3.76
leucine[d]	6.91	7.05
tyrosine[b]	2.94	3.19
phenylalanine	4.14	3.38
histidine	2.10	2.39
lysine	6.89	7.60
arginine	5.03	4.18
tryptophan[e]	2.52	1.08
carbohydrate[f]		
sialic acid	1.4%	
hexose	4.3%	
hexosamines	5.6%	
total	11.3%	
reducing sugars	8.6%	

[a] Determined from triplicate samples after 24, 48, and 72 h of hydrolysis. [b] Extrapolated to zero time hydrolysis. [c] Determined after performic acid oxidation. [d] Values for 72-h hydrolysis time. [e] Determined spectrophotometrically in 6 M Gdn·HCl (Edelhoch, 1967). [f] Refer to Experimental Procedures for details.

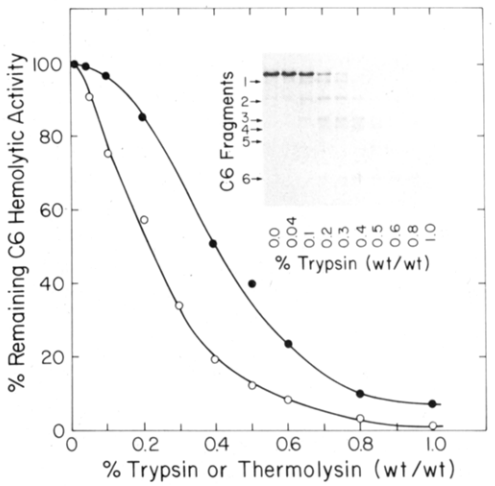

FIGURE 6: Proteolytic digestion of C6. Samples (100 μg) of highly purified C6 were incubated with 0.02–1 μg of trypsin or thermolysin in a total volume of 100 μL of veronal buffered saline for 30 min at 37 °C. A 10-μL portion of each sample was diluted in 40 mL of ice-cold GBV and assayed for remaining C6 hemolytic activity as outlined under Experimental Procedures. Another 10-μL portion of each sample was immediately added to 20 μL of Laemmli slab gel buffer containing 5 mM PMSF and 1% 2-ME and analyzed on a Laemmli $NaDodSO_4$–7.5–15% exponential gradient polyacrylamide slab gel (insert).

extreme care was taken to ensure that the observed inhibition was due to a direct affect on the C6 molecule and not on the assay system. Therefore, the C6 concentrations employed in the initial C6 inhibitor incubation mixtures were diluted 1:10 000 in the final C6 hemolytic assay. Furthermore, in each experiment, increasing concentrations of inhibitor alone were incubated in parallel, all samples were diluted 1:2000 in GVB,

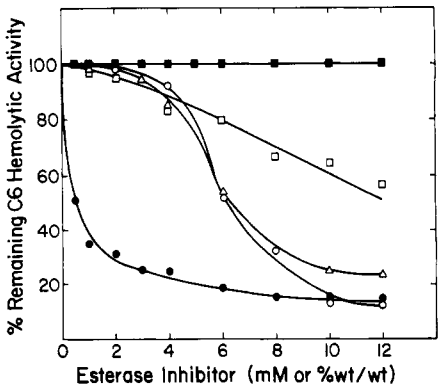

FIGURE 7: Serine esterase inhibitors. Highly purified C6 (4×10^{-7} M) was incubated with the indicated final concentrations of serine esterase inhibitors in VB, pH 7.4, at 37 °C for 3 h. A 10-μL portion of each sample was diluted in 40 mL of ice-cold GVB and assayed for remaining C6 hemolytic activity. DIFP (●); TLCK (○); PMSF (△); dansyl fluoride (□); soybean trypsin inhibitor (concentrations are presented as % w/w) (■).

100 μL of each inhibitor alone dilution was mixed with 100 μL of the nontreated C6 control dilution, and the mixtures were assayed for remaining C6 hemolytic activity. In addition, C6 was also incubated in parallel with increasing volumes of the solvents utilized to prepare the inhibitor stock solutions (methanol or 2-propanol) and assayed for remaining C6 hemolytic activity. In all cases, dilutions of the C6 plus inhibitor experimental incubations were more than sufficient to avoid inhibitor interference with the hemolytic activity assay system, and inhibitor volumes were kept below 10% to avoid solvent effects on the C6 molecule.

The recently described, irreversible inhibitor of serine proteases, pAPMSF (Laura et al., 1980), was examined for its ability to inhibit C6 hemolytic activity. As shown in panel a of Figure 8, pAPMSF at pH 5.9 caused a 50% reduction in C6 hemolytic activity at a concentration of 90 μM, which represented a pAPMSF to C6 molar ratio of 220:1. The inhibition of C6 hemolytic activity by pAPMSF followed first-order kinetics (Figure 9). In addition, C6 was fully protected from pAPMSF inactivation by the synthetic substrate AGLMe (Figure 8, panel b). AGLMe (5×10^{-4}M) was preincubated with 1.75×10^{-4} M pAPMSF for 20 min before the addition of C6 in order to determine that AGLMe protection was not due to a direct interaction with the pAPMSF inhibitor. Preincubation with the synthetic substrate had no effect on the ability of pAPMSF to inhibit C6 as compared to a reaction mixture that had not been preincubated (Figure 9). Additional studies further indicated that AGLMe (1–50 mM) was not able to significantly protect C6 from inactivation by 4 mM $HgCl_2$ or 10 mM pCMB (data not shown).

Discussion

Immunoadsorbent column chromatography has been employed in these studies to provide a rapid, reproducible procedure for the isolation of highly purified C6 in excellent yield with the concomitant production of a C6-depleted reagent (C6D) that can be utilized for the detection and quantitation of nanogram quantities of functionally active C6. The three-step isolation procedure can be performed in 7–9 days with final C6 yields of 40–56% and 1780-1940-fold purification values based on recovery of hemolytic activity for 25 individual preparations. Employing these purification values, using the total protein concentrations of the outdated NHS utilized as the starting material for these preparations (52–73 mg/mL), and assuming the final preparations represented fully hemolytically active C6, we calculated the concentration of C6 in NHS to be 31–43 μg/mL.

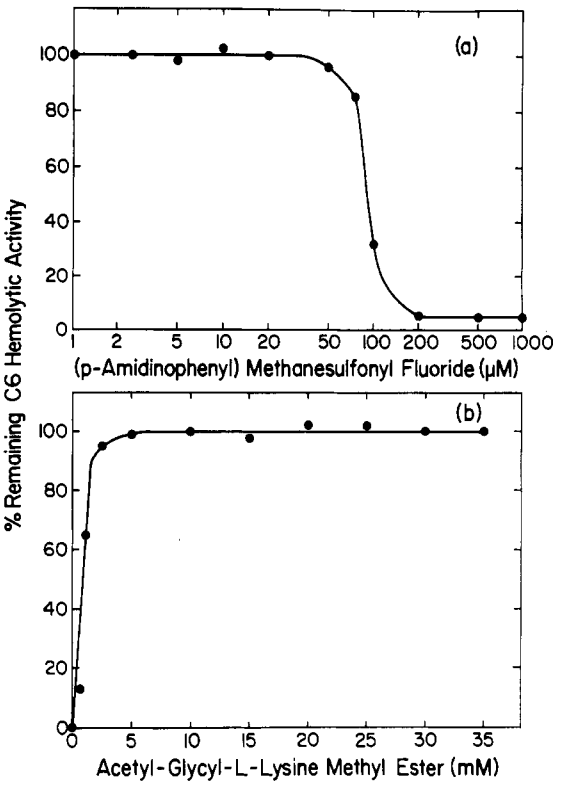

FIGURE 8: Inhibition of C6 by pAPMSF. (a) Highly purified C6 (4×10^{-7} M) was incubated with the indicated concentrations of pAPMSF in VB, pH 5.9, at 45 °C for 1 h. (b) C6 (4×10^{-7} M) was incubated with 1.75×10^{-4} M pAPMSF and the indicated concentrations of the synthetic substrate AGLMe in VB, pH 5.9, at 45 °C for 1 h. A 20-μL portion of each sample was diluted in 25 mL of GVB and assayed for remaining C6 hemolytic activity.

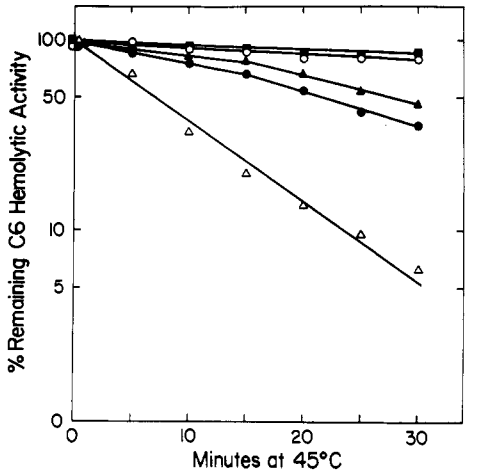

FIGURE 9: Kinetics of pAPMSF inactivation of human C6. Mixtures of 1.75×10^{-4} M pAPMSF in the presence or absence of 5×10^{-4} or 5×10^{-3} M AGLMe were preincubated in VB, pH 5.9, for 20 min at 45 °C before the addition of prewarmed C6 (the final C6 concentration was 4×10^{-7} M). At the indicated time points, 20-μL portions of each sample were diluted in 25 mL of ice-cold GVB and assayed for remaining C6 hemolytic activity. C6 plus 5×10^{-3} M AGLMe alone (■); C6 plus 1.75×10^{-4} M pAPMSF alone (△); C6 plus 1.75×10^{-4} M pAPMSF and 5×10^{-4} M AGLMe (●); C6 plus 1.75×10^{-4} M pAPMSF and 5×10^{-3} M AGLMe (○); C6 plus 1.75×10^{-4} M pAPMSF and 5×10^{-4} M AGLMe without preincubation (▲).

The biochemical characterization data revealed C6 to be a stable serum glycoprotein containing 11.3% carbohydrate and five to six free sulfhydryl groups (cysteine amino acids)

per molecule. This conclusion was based on the specific binding of [^{14}C]pCMB (Riordan & Vallee, 1972) and DTNB (Habeeb, 1972) to highly purified C6 under experimental conditions resulting in saturation of all free sulfhydryl binding sites (Figure 5). However, inactivation of C6 by sulfhydryl reagents appears to be the result of additive, structural perturbations rather than the affinity labeling of a highly reactive, singly important sulfhydryl moiety since an average of 3 mol of pCMB must bind per mol of C6 to achieve functional inactivation.

The major new finding of the present study was the observation that C6 functional activity was inhibitable by a series of serine protease inhibitors, i.e., DIFP, PMSF, and TLCK, over a concentration range of 10^{-2}–10^{-3} M. The most definitive data in this regard were obtained with the recently described, irreversible inhibitor of serine proteases, pAPMSF (Laura et al., 1980). pAPMSF was designed and synthesized to be a highly specific inhibitor of serine proteases by considering the three-dimensional computer-graphic representation of the active site of trypsin. Thus, pAPMSF is an active site directed titrant that irreversibly inactivates a class of plasma serine proteases which demonstrate substrate specificity for the positively charged side chains of the amino acids lysine or arginine by sulfonation of the active-site serine. pAPMSF, at a 5- to 10-fold molar excess over enzyme, can completely inactivate trypsin, thrombin, plasmin, Factor Xa, $C1\bar{r}$ and $C1\bar{s}$ at pH 6.0 in 10–15 min (Laura et al., 1980). However, pAPMSF in large molar excess does not inhibit chymotrypsin or acetylcholinesterase (Laura et al., 1980). Therefore, the ability of pAPMSF to irreversibly inhibit C6 functional hemolytic activity strongly suggests that C6 is a serine protease with a substrate specificity which is similar to that described previously for other complement enzymes, i.e., for the positively charged side chains of lysine or arginine. This conclusion was further supported by the observations that C6 is able to cleave the synthetic substrate AGLMe (W. P. Kolb, unpublished results), and AGLMe has the ability to fully protect C6 from pAPMSF inactivation (Figures 8b and 9).

The naturally occurring C6 substrate is presently unknown. However, in view of the data presented in this report, we are assuming the C6 substrate is either a membrane-associated protein or one of the other terminal complement components. When the mode of action of the other complement enzymes is considered (Müller-Eberhard, 1975; Götze, 1975), the latter possibility would seem the most likely, albeit the report of Kolb & Müller-Eberhard (1975) concluded that the proteolytic cleavage of C5 to C5b represented the last enzymically mediated step in either C pathway. This conclusion was based upon the observation that C5 was contained in the SC5b-9 complex as C5b, as evidenced by the appropriate molecular weight reduction of C5, while the molecular weights of the other C proteins in the SC5b-9 complex were indistinguishable from those of their respective precursors. However, in retrospect, the study of Kolb & Müller-Eberhard (1975) may not have been exhaustive enough to exclude the possibility that an enzymatic step was required for the expression of attack complex membranolytic activity for the following reasons: (1) The disc-gel system employed was not able to detect relatively small changes (<5%) in the precursor molecular weights subsequent to complex formation, (2) the $NaDodSO_4$ disc-gel analyses were conducted only under nonreducing conditions, and (3) only SC5b-9 complexes were studied, but membrane solubilized, MC5b-9 complexes were not examined. This latter point is of particular interest because the SC5b-9 complex, as isolated from C-activated NHS, is a 23S monomeric complex that is hemolytically inactive and contains serum S protein (Podack et al., 1978; Podack & Müller-Eberhard, 1979) and antithrombin III (AT III) (Curd et al., 1978). MC5b-9 complexes are devoid of S protein (Bhakdi et al., 1976; Ware et al., 1981) and AT III (W. P. Kolb et al., unpublished data). Serum S protein binds to nascent C5b,6,7 complexes in fluid phase during the assembly of C5b,6 complex with C7 and thereby inhibits C attack complex binding to membrane surfaces (Podack et al., 1978). AT III is an effective inhibitor of activated serum serine proteases (Rosenberg, 1977). Therefore, it seems likely that the binding of S protein and AT III to SC5b-9 complexes would inhibit or modulate the expression of C6 enzymatic activity. Thus, future investigations concerning the identification of the naturally occurring substrate of C6 should be restricted to MC5b-9 complex assembly events.

In conclusion, the sixth component of human C has been described as an additional C enzyme whose activity is required for C-directed target cell cytolysis. Since a large molar excess of pAPMSF was required for C6 inactivation (220:1, Figure 8a) as compared to $C1\bar{r}$ or $C1\bar{s}$ inactivation (Laura et al., 1980), we are assuming that funtionally active C6 as it is isolated from NHS does not express its full enzymatic potential. Whether C6 is isolated in zymogen form, which requires covalent modification to express full enzymatic activity, or whether the interaction between C6 and activated C5b resulting in C5b,6 complex assembly induces conformational changes in the C6 molecule allowing for full enzymatic expression is currently unknown. Nevertheless, determination of which one of these hypothesis might be correct (we currently favor the latter) and identification of the naturally occurring C6 substrate are obvious prerequisites required for a better biochemical understanding of C membrane attack complex assembly and membranolytic function.

Acknowledgments

We acknowledge that the amino acid analyses were performed at the Protein Chemistry Facility of the University of Texas Health Science Center at San Antonio. We also thank Dr. Steven Mattingly for conducting the carbohydrate analyses and Dr. David Bing for providing the pAPMSF employed in these studies.

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Stable, Soluble, Model Immune Complexes Made with a Versatile Multivalent Affinity-Labeling Antigen†

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ABSTRACT: We describe here the synthesis of a family of multivalent affinity-labeling antigens based on the soluble carbohydrate polymer Ficoll. Ficoll was derivatized successively with chloroacetate, ethylenediamine, and glutaric anhydride and finally esterified with 2,4-dinitrophenol. Prior to esterification, the polymer could also be derivatized with tyramine to allow trace iodination and with the monosaccharides galactose or mannose. The numbers of substituent groups could be controlled at several points in the synthesis. The resulting multiple dinitrophenyl esters on a Ficoll or glycosylated Ficoll polymer specifically cross-linked anti-dinitrophenyl antibodies to form covalently cross-linked antigen–antibody complexes. The glycosylated Ficolls were particularly made for studies of the influence of antigen structure on the behavior of immune complexes. The intermediates in the synthesis are suitable for other derivatizations as well. These model immune complexes are stable and soluble, they can be separated by size, and they overcome some of the limitations on the study of complexes imposed by previous techniques of preparing them.

Immune complexes appear to be an important cause of tissue injury in many illnesses. Studying the mechanism of their effects has been limited by several factors, however. First, because the interactions between antigens and antibodies are noncovalent, the manipulations necessary to study them in vitro can alter the antigen–antibody complex. Second, since the antigen in the complex is usually not known, it is not possible to ask precise questions about the properties of the complex. Third, complexes found in the circulation may be pathologenetically irrelevant since they are representative of those very complexes which have not settled in the tissues to cause injury.

Several approaches to overcome these limitations have involved attempts to produce stable (covalently cross-linked) antigen–antibody complexes. Heat-aggregated γ-globulin has been used as a model of antibodies aggregated by antigen (Christian, 1960; Ishizaka et al., 1967; Knutson et al., 1977). Although some properties of such aggregates resemble the properties of antigen–antibody complexes, the aggregation induced by heat imperfectly resembles that induced by antigen in being nonspecific as well as difficult to control. Its failure to provide the proper orientation is demonstrated by the sometimes striking differences between the behavior of heat-aggregated immunoglobulin and immune complexes. Two other nonspecific methods of aggregation have yielded useful observations: cross-linking by dimethyl suberimidate of immunoglobulins in solution (Segal et al., 1977) and by

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