

EXPRESSION OF C5a-LIKE BIOLOGICAL ACTIVITIES BY THE
FIFTH COMPONENT OF HUMAN COMPLEMENT (C5) UPON
LIMITED DIGESTION WITH NONCOMPLEMENT ENZYMES
WITHOUT RELEASE OF POLYPEPTIDE FRAGMENTS*

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The fifth component of complement (C5)¹ is a 210,000-mol wt plasma glycoprotein comprised of two polypeptide chains (α and β) that are linked together by disulfide bonds and noncovalent interactions (1). The C5 α and β subunits possess molecular weights of 115,000 and 75,000, respectively (2). Upon activation of either complement reaction sequence the C5 convertase enzymes, C4b,2a,3b (3-5) or C3b_nBb,P (6), hydrolyze a specific peptide bond within the C5 α -chain resulting in the production of C5a and C5b fragments. The smaller C5a fragment (C5a anaphylatoxin) is a cationic peptide derived from the first 74 amino acid residues of the amino-terminus of the C5 α -subunit (2, 7-10). The primary amino acid sequence of human (11, 12) and porcine (13, 14) C5a has been determined. The C5a anaphylatoxin expresses a wide variety of biological activities including (a) contraction of smooth muscle (15-18), (b) degranulation of mast cells (7, 19), (c) chemotaxis of polymorphonuclear neutrophils (PMN) (7, 18, 20, 21), and (d) secretion of azurophilic granular enzymes from PMN (22-26). Expression of C5a biological activity is regulated by the endogenous plasma enzyme carboxypeptidase N (E.C.3.4.12.7), which rapidly removes the carboxy-terminal arginine from C5a, producing the C5a des Arg derivative that exhibits greatly reduced activity (25-27). The restricted C5a des Arg anaphylatoxin activity can be enhanced by a serum-derived "cochemotaxin" (28, 29), or in the case

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¹ *Abbreviations used in this paper:* C5, fifth component of complement; β ME, β -mercaptoethanol; EDTA, ethylenediamine tetraacetate; HBSS, Hank's balanced salt solution, pH 7.4, containing 1.3 mM CaCl₂, 5.4 mM KCl, 0.4 mM KH₂PO₄, 0.5 mM MgCl₂·6H₂O, 0.4 mM MgSO₄·7H₂O, 140 mM NaCl, 4.2 mM NaHCO₃, 0.3 mM Na₂HPO₄·7H₂O, and 5.6 mM D-glucose; HBSS-0.1% BSA, Hank's balanced salt solution containing 1 g/l bovine serum albumin; HBSS-0.3% BSA, Hank's balanced salt solution containing 3 g/l bovine serum albumin; Hepes, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; LDH, lactate dehydrogenase; NHS, normal human serum; PBS, phosphate-buffered saline, pH 7.4, containing 5 mM phosphate and 145 mM NaCl; PBSE, PBS containing 10 mM EDTA; PMN, polymorphonuclear neutrophils; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide slab gel electrophoresis; VB, isotonic veronal-buffered saline, pH 7.4, containing 5 mM barbital, 145 mM NaCl, 0.15 mM CaCl₂, and 0.5 mM MgCl₂.

of human C5a des Arg, by removal of the oligosaccharide moiety (30, 31).

A number of physiologically relevant, non-complement-related, proteolytic enzymes also are able to activate C5 to express C5a-like biological activities that can initiate or potentiate acute inflammatory reactions. For example, trypsin (21, 32-34), α -thrombin (35), elastase (36), and cathepsin G (37) can modify C5 to express PMN chemotactic and lysosomal enzyme-releasing activities. Since these enzymes are able to activate C5 to express biological activities that are indistinguishable from C5a-mediated reactions, most investigators have concluded that noncomplement protease enzymes produce C5a, or a C5a-related fragment, which is released subsequent to limited hydrolysis of C5 (15, 22, 35). However, our recent reports (38, 39) indicate that limited trypsin digestion of C5 resulted in the expression of C5a-like biological activities without the production of C5a and C5b fragments. To further document and characterize the mechanism by which noncomplement enzymes are able to activate C5 to express C5a-like biological activities, we report that (a) the first C5 α -chain site of trypsin hydrolysis is responsible for the activation of C5 to express human neutrophil chemotaxis and lysosomal enzyme-releasing activity, (b) the trypsin-activated form of C5, which has a molecular weight of 210,000, retains biological activity even after preincubation and gel filtration column chromatography in the presence of sodium dodecyl sulfate (SDS), (c) α -thrombin, elastase, and plasmin also are able to activate C5 to express PMN lysosomal enzyme-releasing activity, and (d) the mechanism of C5 activation by α -thrombin is similar to the novel activation mechanism described for trypsin.

Materials and Methods

Purified Complement Components and Reagents

PURIFIED HUMAN C5. Highly purified C5 was isolated from outdated human plasma as described previously (40) with the following modifications. Instead of using QAE-Sephadex as the final chromatographic step, the contaminant proteins present in the C5 preparations after anti-C5 immunoadsorbent column chromatography were removed after dialysis by passage through an anti-C5 impurities immunoadsorbent column as similarly described for the isolation of C6 (41). Antisera to contaminant proteins present in the C5 preparations (anti-impurities antisera) were raised in goats by injection of the C5-depleted, nonadsorbed protein fractions obtained after passage of the resuspended 37% ammonium sulfate precipitate of normal human serum (NHS) (C5 starting material) through the anti-C5 immunoadsorbent column. The IgG fraction from 500 ml of anti-impurities antisera was prepared by standard procedures (42) 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. (43) as described by Kolb et al. (44).

RADIOIODINATION. High purified human C5 was radiolabeled with Na¹²⁵I by the solid-phase, mixed glucose oxidase, lactoperoxidase procedure as described previously (38).

AFFINITY PURIFICATION OF CAPRINE ANTI-HUMAN C5 ANTIBODIES. The C5 immunoadsorbent was prepared by covalently coupling 25 mg of highly purified human C5 to CNBr-activated Sepharose CL-4B at 1 mg C5/ml of beads as described previously (44). 50 ml of monospecific caprine anti-human C5 (IgG) was applied to the C5 immunoadsorbent column at a flow rate of 1 ml/h. The column was washed with 50 ml phosphate-buffered saline (PBS) containing 10 mM EDTA (PBSE) followed by 50 ml PBSE containing 2 M NaCl at a flow rate of 5 ml/h. The anti-C5 antibodies were eluted with 25 ml of 0.2 M sodium phosphate buffer, pH 6.0, containing 2 M KSCN at a flow rate of 20 ml/h collecting 2-ml fractions. The affinity-purified anti-C5 antibody containing fractions were pooled, exhaustively dialyzed against Hanks' balanced salt solution (HBSS), concentrated by positive pressure ultrafiltration, filter sterilized, and stored at -70°C in 100- μ l portions.

PROTEASES. Human plasmin, kallikrein, and elastase, as well as bovine pancreatic trypsin

were purchased from Sigma Chemical Co., St. Louis, MO. Highly purified human α -thrombin was provided by Dr. J. W. Fenton (New York State Dept of Health, Albany, NY) (45). The trypsin was purified further by affinity column chromatography as described by Robinson et al. (46), as modified by Wetsel and Kolb (38). After elution from the affinity column, the trypsin was dialyzed against 1 mM HCl and stored at -70°C .

SDS-Polyacrylamide Slab Gel Electrophoresis. Polyacrylamide slab gel electrophoresis in the presence of SDS (SDS-PAGE) was conducted as described by Laemmli (47) using a 5% acrylamide stacking gel. Nonreduced samples were run on 7% acrylamide running gels and reduced samples were run in the presence of 1% β -mercaptoethanol (β ME) on 10% acrylamide running gels. After electrophoresis, the slab gels were fixed in 10% trichloroacetic acid-20% methanol, stained in 25% methanol-10% acetic acid containing 0.25% Coomassie Blue, and destained in 10% isopropanol-10% acetic acid.

The C5 α -chain fragments, generated by trypsin cleavage, were quantitated from reduced SDS-PAGE slab gels using a Helena Quick-Scan R and D electrophoresis densitometer (Helena Laboratories, Beaumont, TX). The peak areas of stained C5 α -chain fragment protein bands were calculated by using a Carl Zeiss MOP-3 digitometer. Corrections for small differences in the amount of protein applied to each gel lane were made by normalizing the relative molar amounts of C5 α -chain fragments in each lane to the relative molar amount of C5 β -chain present in each lane.

Isolation of Human Polymorphonuclear Neutrophils. Human polymorphonuclear neutrophils (PMN) were isolated from healthy volunteers by the procedure of Boyüm (48) with modifications as described previously (38).

Polymorphonuclear Neutrophil Chemotaxis and Lysosomal Enzyme-releasing Assays. Protease-activated human C5 was assayed for PMN chemotactic and lysosomal enzyme-releasing activities as described previously (38).

Protein Determinations. Protein concentrations of purified human C5 were determined by the Folin procedure of Lowry et al. (49) using a C3 standard curve. Protein concentrations of the purified trypsin solutions were determined spectrophotometrically at 280 nm utilizing an $E_{1\text{cm}}^{1\%}$ of 15.4 (46).

Results

Expression of C5a-Like Biological Activity by Trypsin-modified C5

DEPENDENCE ON THE MOLAR CONCENTRATION OF C5 IN THE C5 ACTIVATION MIXTURE. Increasing molar concentrations of C5 were incubated with a fixed molar ratio of affinity-purified trypsin (0.008 mol trypsin/mol C5) in VB at 37°C for 30 min. Portions of each sample were removed, diluted in HBSS containing 1 g/liter bovine serum albumin (HBSS-0.1% BSA) containing 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor (SBTI), and 0.5 μg of trypsin-digested C5 from each dilution was assayed in duplicate for PMN lysosomal enzyme-releasing activity as described in Materials and Methods. As illustrated in Fig. 1, the amount of lysosomal enzyme-releasing activity generated by trypsin activation of C5 was dependent upon the C5 molar concentration present in the initial digestion reaction. Maximum lysosomal enzyme-releasing activity was generated when C5 was present in the proteolysis reaction mixture at a concentration of 5×10^{-6} M (1 mg/ml). C5 concentrations less than or greater than 5×10^{-6} M resulted in decreased activity, presumably due to insufficient or excessive C5 hydrolysis, respectively. Therefore, unless stated otherwise, all subsequent C5 activation assays used 1 mg C5/ml of VB at 37°C for 30 min.

DOSE RESPONSE. Highly purified human C5 (1 mg/ml) was incubated with 0.008 mol of trypsin/mol C5 as described above. After a 30-min incubation at 37°C , samples were removed and serially diluted in either HBSS-0.1% BSA containing 10 μg SBTI/ml or HBSS-0.3% BSA containing 10 μg SBTI/ml. Increasing concentrations

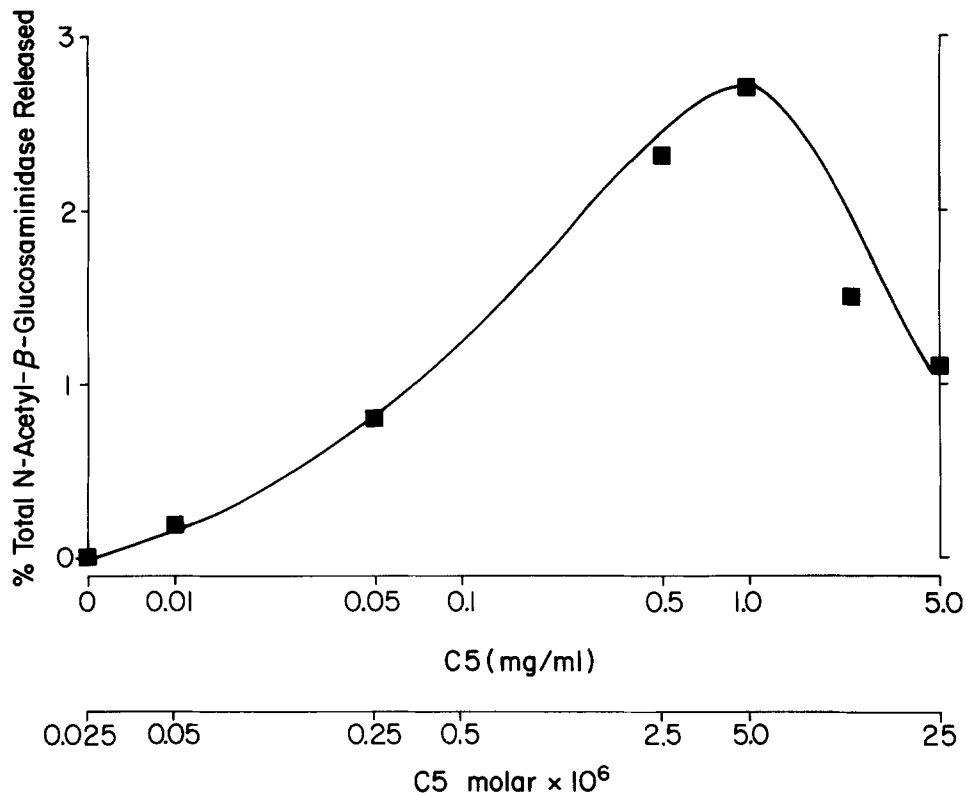


FIG. 1. Determination of the C5 molar concentration required for optimal activation by trypsin. Purified human C5, at the indicated molar concentrations, was incubated with a constant molar ratio of trypsin as detailed in the text. After activation, 0.5 μ g of each trypsin-digested C5 incubation mixture was assayed for PMN lysosomal enzyme-releasing activity.

of trypsin-activated C5 were assayed for PMN lysosomal enzyme-releasing and chemotactic activities as outlined in Materials and Methods. The results (Fig. 2) indicated that trypsin-treated C5 effected the release of PMN lysosomal enzymes in a dose-dependent manner over a concentration range of 1×10^{-12} to 10×10^{-12} mol per assay mixture (4×10^6 PMN). However, a linear dose response was demonstrated only at inputs of trypsin-activated C5 $< 3 \times 10^{-12}$ mol (0.6 μ g) (Fig. 2, panel A). The chemotaxis assay proved to be more sensitive with trypsin-activated C5 effecting PMN chemotaxis in a dose-dependent manner over a concentration range of 0.5×10^{-13} to 10×10^{-13} mol per assay chamber (1×10^6 PMN). A linear dose response was demonstrated only at inputs of trypsin-activated C5 $< 3 \times 10^{-13}$ mol (0.06 μ g) (Fig. 2, panel B). Therefore, unless stated otherwise, subsequent PMN lysosomal enzyme-releasing and chemotactic assays were monitored in their linear dose response ranges by utilizing 0.5 or 0.05 μ g of trypsin-activated C5 per assay, respectively.

SITE OF C5 α -CHAIN HYDROLYSIS GENERATING BIOLOGIC ACTIVITY. Highly purified human C5 was incubated with affinity-purified trypsin (0.008 mol trypsin/mol C5) at 37°C at a final C5 concentration of 1 mg/ml isotonic veronal-buffered saline (VB). After various time intervals, triplicate samples were collected simultaneously. Phenylmethylsulfonyl fluoride (PMSF) was immediately added to the first set of samples

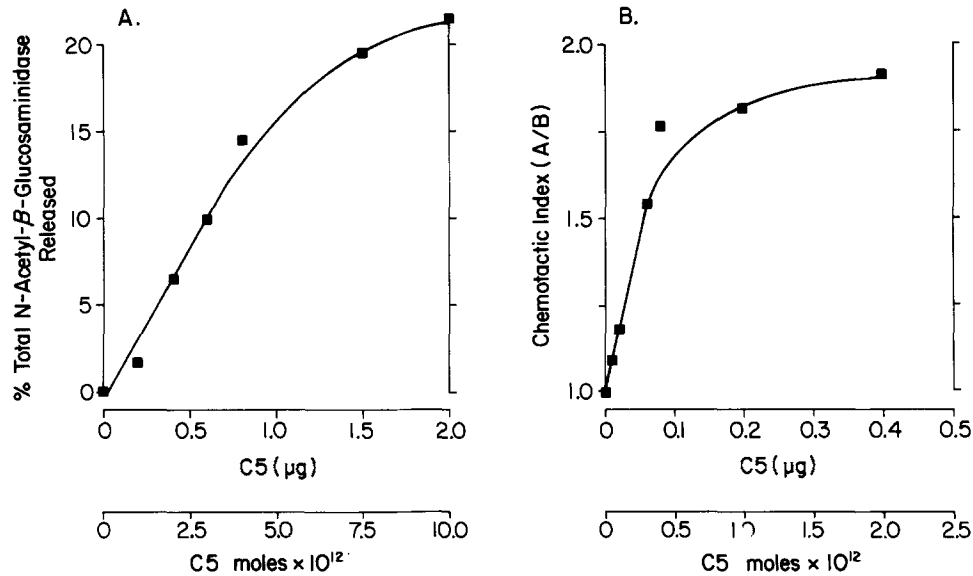


FIG. 2. Dose response assays of C5 activated by trypsin under optimal conditions. Increasing concentrations of C5 activated by trypsin under optimal incubation conditions, as determined from the results in Fig. 1, were assayed for the ability to mediate neutrophil lysosomal enzyme secretion and chemotaxis as outlined in Materials and Methods. Undigested C5 expressed no biological activity over the concentration ranges examined.

(10 μ l each) to a final concentration of 2.5 mM, and after collection of all time points, this first set of samples was analyzed by SDS-polyacrylamide slab gel electrophoresis under reducing conditions. To substantiate the ability of PMSF to completely inhibit trypsin digestion, PMSF was added to highly purified C5 (1 mg/ml VB) to a final concentration of 2.5 mM before the addition of trypsin (0.008 mol trypsin/mol C5), which was followed by a 30-min incubation at 37°C and SDS-PAGE analysis (Fig. 3, upper panel, lane C). After protein staining with Coomassie Blue, the SDS-polyacrylamide slab gels were analyzed on a scanning densitometer and the relative molar amounts of the trypsin-produced C5 α -chain fragment, as defined previously (38), were quantitated and normalized by the molar amount of C5 β -chain present in each gel lane as outlined in Materials and Methods. The second set of time point samples (5 μ l each) were diluted immediately into 3 ml of HBSS-0.1% BSA containing 10 μ g SBTI/ml, and 2.4×10^{-12} mol (0.5 μ g) of C5 from each were assayed for PMN lysosomal enzyme-releasing activity. The third set of time point samples (5 μ l each) were diluted immediately into 3 ml of HBSS-0.3% BSA containing 10 μ g SBTI/ml, and 2.4×10^{-13} mol (0.05 μ g) of C5 from each were assayed for PMN chemotaxis. As seen in Fig. 3, the first trypsin-mediated C5 cleavage event, i.e., C5 α -chain conversion to C5 α_1 and C5 α_5 (C5') was responsible for activating trypsin-treated C5 to express both PMN lysosomal enzyme-releasing and chemotactic activities.

The Question of a Released Fragment(s) from Trypsin-modified C5 Expressing C5a-Like Biological Activity

KINETICS OF C5 ACTIVATION BY TRYPSIN. To examine the possible production and release of a biologically active fragment from trypsin-modified C5, the kinetics of C5 activation by trypsin was determined. Highly purified human C5 at a concentration

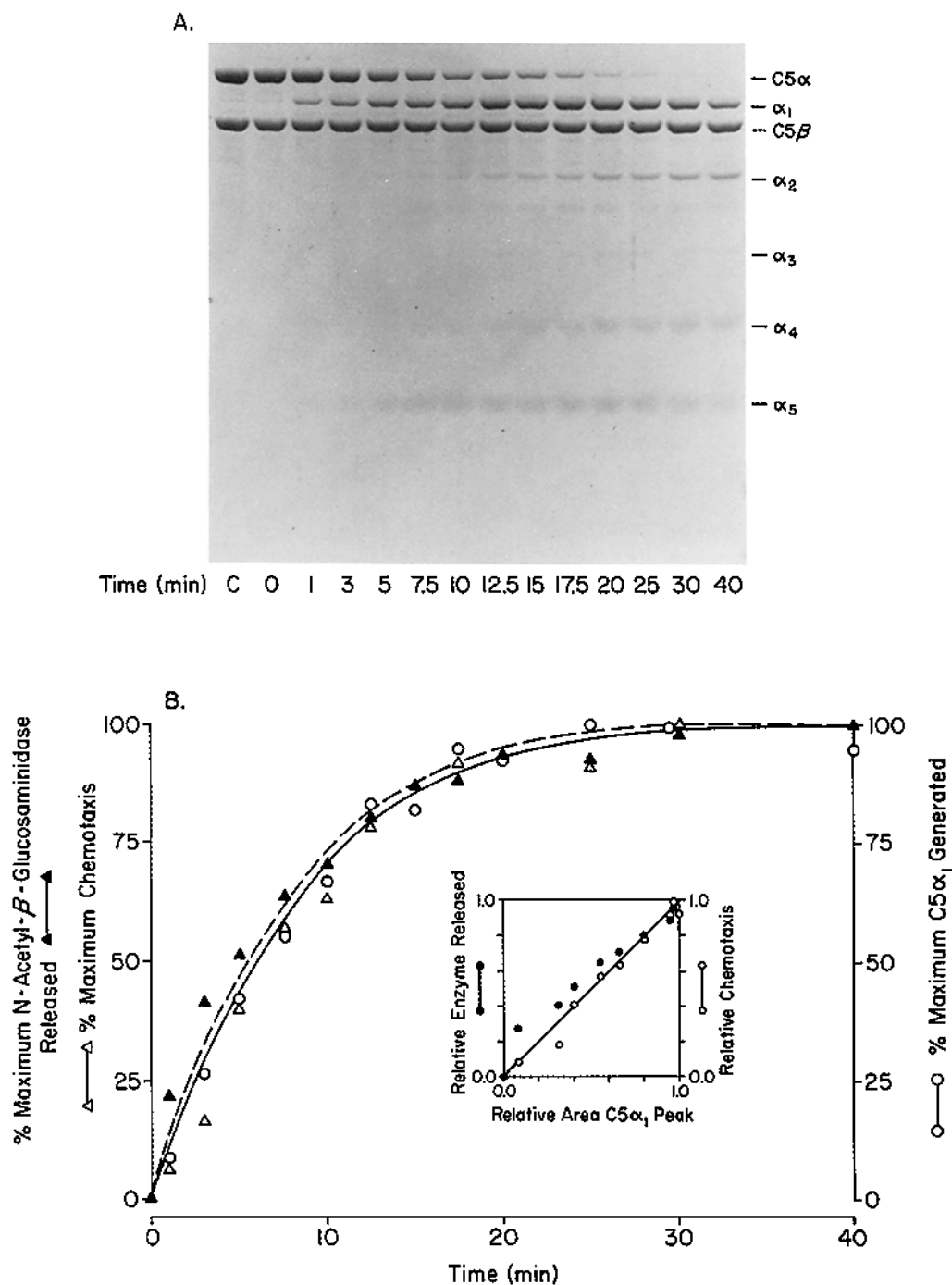


FIG. 3. Correlation between the site of C5 α -chain cleavage and acquisition of biological activity. Highly purified C5 was incubated at 37°C with 0.008 mol trypsin/mol C5 at a final C5 concentration of 1 mg/ml (5×10^{-6} M). Samples were removed at the indicated time intervals and the amount of C5 α -chain hydrolysis (A) and PMN lysosomal enzyme-releasing and chemotactic activities were quantitated as outlined in the text. In B, the percent of maximum C5 α_1 fragment produced, N-acetyl- β -glucosaminidase released, and chemotactic activity generated were plotted as a function of time. When the relative amounts of biological activity generated were plotted as a function of the relative molar amounts of the C5 α_1 fragment produced at each time point, correlation coefficients of 0.960 and 0.945 were obtained for the PMN chemotactic and enzyme releasing activity plots respectively (B, inset).

of 1 mg/ml VB was incubated with 0.03 mol of affinity-purified trypsin/mol of C5 at 37°C. After various time intervals, 5- μ l samples were diluted into 3 ml of HBSS-0.1% BSA containing 10 μ g SBTI/ml, and 2.4×10^{-12} mol (0.5 μ g) of each trypsin-treated sample were assayed for PMN lysosomal enzyme-releasing activity. Detectable lysosomal enzyme-releasing activity was observed after 1 min of incubation, and 100% of maximum activity was generated after 20 min of incubation. Incubation periods longer than 20 min resulted in a time-dependent decrease in the expression of trypsin-modified C5 biological activity with only 10% of maximum activity remaining after 150 min (data not shown).

GEL FILTRATION AT PHYSIOLOGIC IONIC STRENGTH, pH 7.4. 2 mg of ^{125}I -C5 were incubated with 0.03 mol trypsin/mol C5 in a final volume of 2 ml VB at 37°C for 20 min, which resulted in the expression of 100% of maximum PMN lysosomal enzyme-releasing activity as outlined above. Another 2-mg sample of ^{125}I -C5 was incubated with 0.03 mol trypsin/mol C5 in a final volume of 2 ml VB at 37°C for 100 min, which resulted in the expression of only 30% of maximum PMN lysosomal enzyme-releasing activity due to excessive trypsin-digestion. Both trypsin-activated ^{125}I -C5 samples were made 2.5 mM in PMSF and subjected to Biogel P-200 (Bio-Rad Laboratories, Richmond, CA) gel filtration column chromatography. As seen in Fig. 4, panel A, the trypsin-activated ^{125}I -C5 sample expressing 100% of maximum activity eluted at the same V_e/V_t as native ^{125}I -C5 with a corresponding molecular weight of 210,000. In addition, all detectable PMN lysosomal enzyme-releasing activity was associated with only the high molecular weight, trypsin-modified C5-containing fractions. However, the excessively digested ^{125}I -C5 sample expressing only 30% of maximum activity yielded two additional C5-derived fragment peaks with molecular weights of $\sim 15,000$ and 5,000 (Fig. 4, panel B). Although the 15,000 molecular weight fragment expressed a limited amount of lysosomal enzyme-releasing activity when 300- μ l portions of the column fractions were assayed (six times the volume routinely employed), over 95% of the C5a-like biological activity was associated with the 210,000 molecular weight form of trypsin-activated C5.

The extent of C5 digestion that had occurred in these experiments was determined upon SDS-polyacrylamide slab gel analysis of the 210,000 molecular weight peak fractions obtained from the two column profiles presented in Fig. 4 as indicated. Under nonreducing conditions (Fig. 5, *left*), the trypsin-treated C5 expressing 100% of maximum activity (pool A) ran as a single protein band with the same molecular weight as native C5, while the trypsin-treated C5 expressing 30% of maximum activity (pool B) was hydrolyzed more extensively as evidenced by the presence of C5I and C5II fragments, as defined previously (38). Upon reduction (Fig. 5, *right*), the biologically active form of the trypsin-activated C5 expressing 100% of maximum activity (pool A) was determined to be C5' since $78 \pm 5\%$ of the C5 α -chain derived protein was present as C5 α_1 and C5 α_5 . In contrast, the extensively trypsin-digested C5 expressing only 30% of maximum activity (pool B) was devoid of a C5 α_1 fragment, which had been hydrolyzed completely to C5 α_2 and C5 α_4 . Quantitative densitometric scans of these gels indicated the relative molar amount of C5 α_5 fragment present in the trypsin-treated C5 expressing 30% of maximum activity was $\sim 30\%$ of the molar amount of C5 α_5 fragment present in the trypsin-treated C5 expressing 100% of maximum activity. Therefore, these results strongly suggested that the determining factor required for the expression of C5a-like biological activities by trypsin-modified

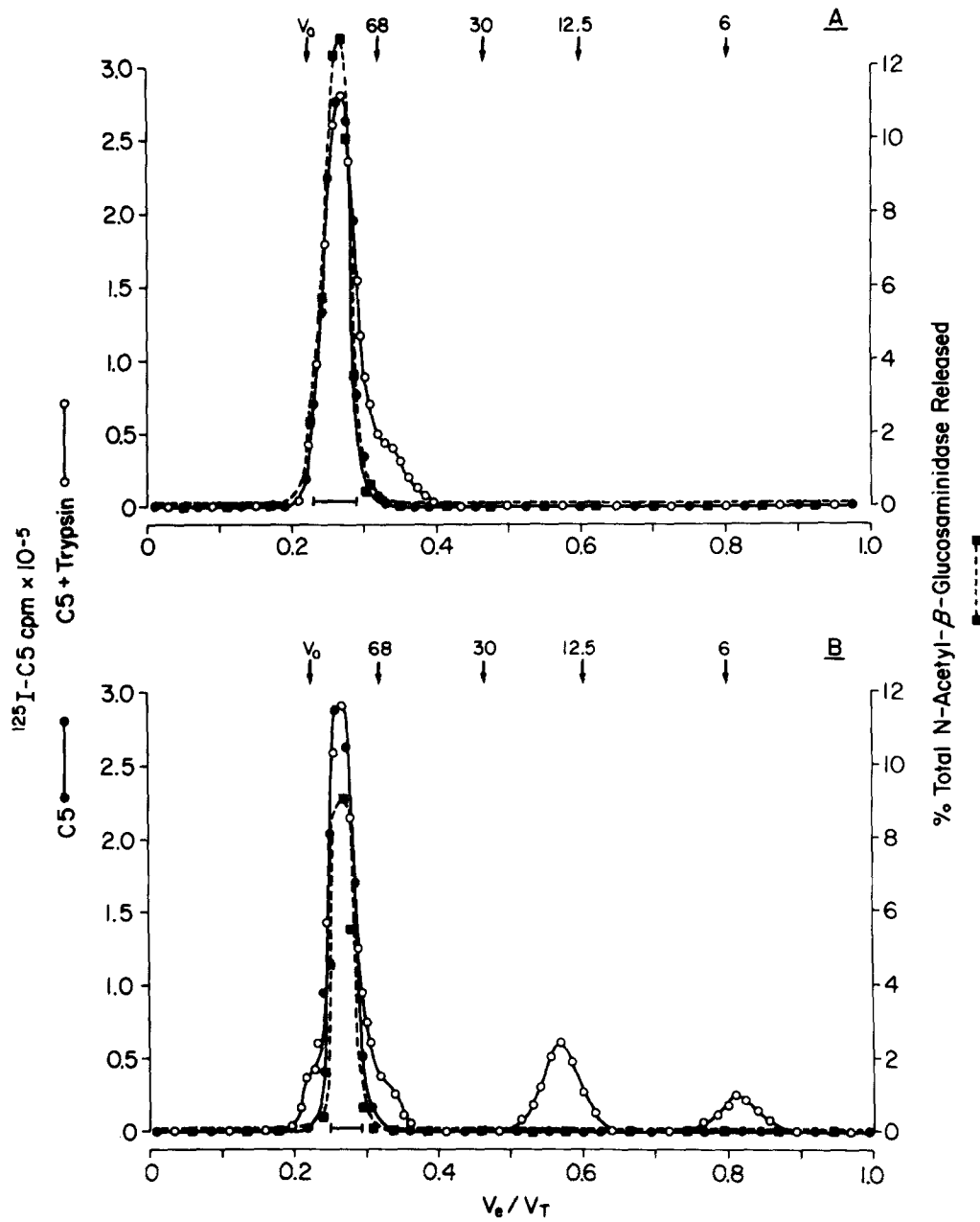


FIG. 4. Gel filtration column chromatography at pH 7.4 of trypsin-activated C5. Human C5 was digested with trypsin for 20 min (A, 100% of maximum activity) or 100 min (B, 30% of maximum activity) as described in the text and subjected to gel filtration column chromatography using Biogel P-200. The column (1.5×60 cm) was equilibrated with PBS and 500- μl samples were collected at a flow rate of 4 ml/h at 4°C . 50- μl portions from the indicated fractions were assayed for PMN lysosomal enzyme-releasing activity. Molecular weight estimates were determined by utilizing the following reference markers and indicated mol wt values ($\times 10^3$): thyroglobulin (void volume, V_0), BSA (68), carbonic anhydrase (30), cytochrome C (12.5), aprotinin (6) and phenol red (total volume, V_T).

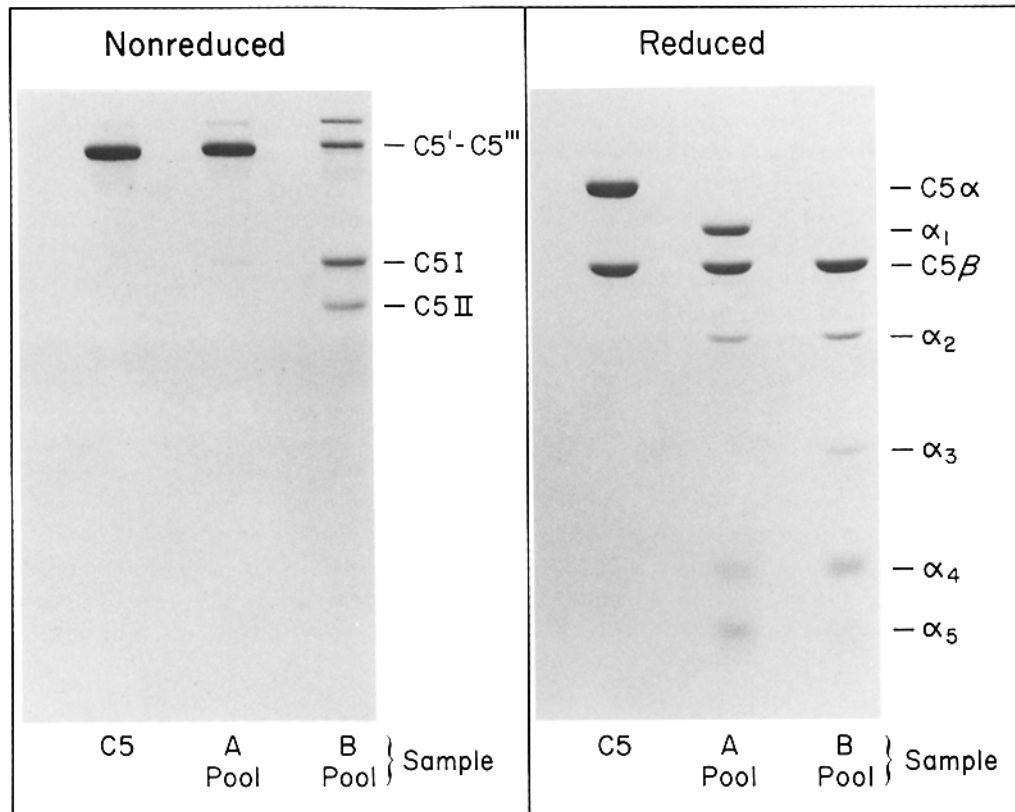


FIG. 5. SDS-polyacrylamide slab gel electrophoresis. The 210,000 mol wt biologically active fractions obtained subsequent to gel filtration analysis of trypsin-activated C5, as seen in Fig. 4, were pooled where indicated and subjected to SDS-polyacrylamide slab gel electrophoresis in the presence or absence of β ME as described in Materials and Methods. The pool obtained from Fig. 4A, was derived from the C5 digest expressing 100% of maximum activity and is labeled Pool A. The pool obtained from Fig. 4B, was derived from the C5 digest expressing 30% of maximum activity and is labeled Pool B.

forms of C5 was the presence of the C5 α_5 fragment.

GEL FILTRATION OF TRYPSIN-ACTIVATED C5 AT pH 4.0. The results presented in Figs. 4 and 5 do not support the previously published report of Cochrane and Müller-Eberhard (15), which concluded that the only biologically active, C5-related component produced upon trypsin digestion of human C5 was the C5a anaphylatoxin fragment, as demonstrated upon gel filtration column chromatography at pH 4.0. Gel filtration was conducted at pH 4.0 in their studies to insure complete dissociation of noncovalently bound fragments from trypsin-activated C5 molecules. In an effort to closely examine and perhaps determine the basis for these apparently conflicting results, 3 mg of ¹²⁵I-C5 was incubated with 60 μ g of trypsin (0.17 mol trypsin/mol C5) in a final volume of 3 ml VB at 32°C for 5 min. These activation conditions were selected to approximate those employed by Cochrane and Müller-Eberhard (15) as closely as possible. The sample was made 2.5 mM in PMSF and adjusted to pH 4.0 by the slow dropwise addition of 2 N HCl. In the majority of experiments (4/6), pH adjustment to 4.0 resulted in overt precipitation of trypsin-activated C5. Analysis of

precipitated or apparently nonprecipitated trypsin-activated C5 at pH 4.0 by Biogel P-200 gel filtration column chromatography yielded similar results. Namely, the 210,000 molecular weight form(s) of trypsin-activated C5 was bound to or precipitated on the gel filtration column at pH 4.0 and could not be eluted (Fig. 6). However, minor amounts of ^{125}I -C5 derived fragments of approximately 5,000 and 10,000 molecular weight were eluted that expressed detectable levels of C5a-like biological activity only when 400- μl portions of the indicated fractions were assayed for PMN lysosomal enzyme-releasing activity; no C5a-like activity was demonstrable when 50- μl portions from these fractions, the volume normally employed to detect C5' activity (Fig. 4A and B), were assayed.

ISOLATION OF TRYPSIN-ACTIVATED C5 IN THE PRESENCE OF SDS. To further substantiate the conclusion that the predominant, biologically active form of trypsin-activated C5 is a 210,000 molecular weight, trypsin-modified C5 molecule (C5'-C5''' as defined previously [38]), and to exclude the possibility that the biological activity expressed by trypsin-activated C5 was due primarily to the production of a small molecular weight, noncovalently bound fragment(s) that could be slowly released, trypsin-activated C5 was preincubated with SDS and subjected to gel filtration column chromatography in the presence of SDS, a strong protein-denaturing and -dissociating detergent. 5 mg of ^{125}I -C5 was incubated with 0.008 mol trypsin/mol C5 at 37°C for 30 min in a total volume of 5 ml VB. Trypsin was removed with chicken ovomucoid-

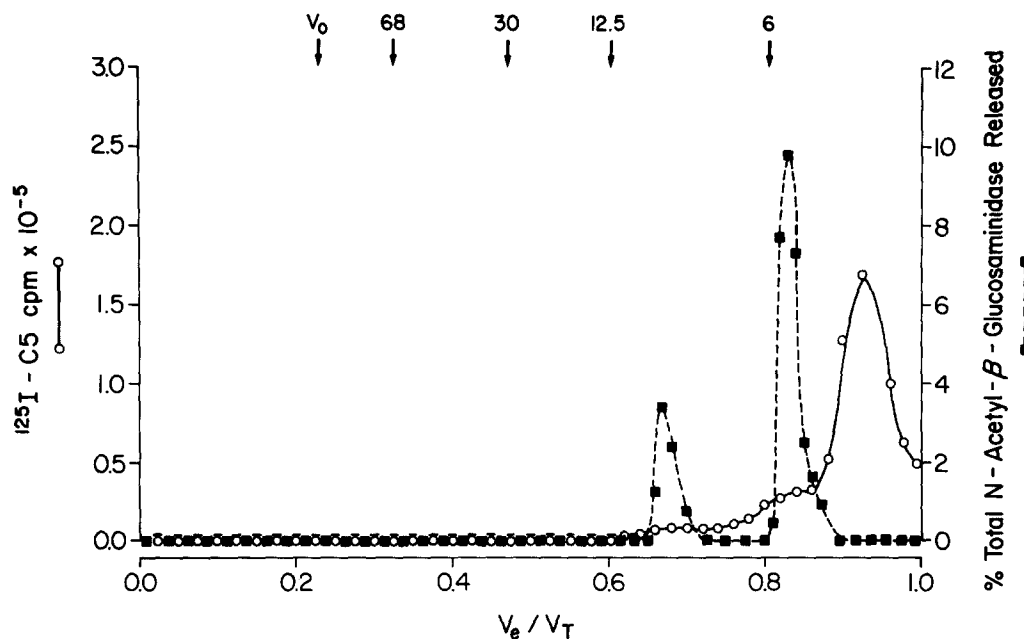


FIG. 6. Gel filtration column chromatography at pH 4.0 of trypsin-activated C5. Human C5 (3 mg) was digested with trypsin (60 μg) for 5 min at 32°C and subjected to gel filtration column chromatography employing Biogel P-200. The column (1.5 \times 100 cm) was equilibrated with 10 mM sodium acetate buffer, pH 4.0, containing 150 mM NaCl and 1-ml fractions were collected at a flow rate of 4 ml/h at 22°C. 400- μl portions from the indicated fractions were assayed for PMN lysosomal enzyme-releasing activity. Molecular weight reference markers were as indicated in the legend to Fig. 4. Under these conditions, the 210,000 molecular weight forms of trypsin-activated C5 precipitated on the column.

Sepharose 4B (46). SDS was added to the trypsin-digested C5 supernatant to a final concentration of 2% (wt/vol), and incubated at 22°C for 60 min. The SDS-treated sample was concentrated to a final volume of 2 ml by ultrafiltration employing a YM-5 Amicon membrane (Amicon Corp., Scientific Sys. Div., Danvers, MA) and subjected to gel filtration analysis utilizing a Sepharose CL-4B column (1.5 cm × 120 cm) equilibrated with PBS containing 0.2% SDS. As seen in Fig. 7, the majority of trypsin-activated ^{125}I -C5 eluted from the gel filtration column as a high molecular weight peak. A minor, lower molecular weight component was also evident (V_e/V_t of 0.81–0.84). The fractions containing the high molecular weight form of trypsin-activated ^{125}I -C5 were pooled as indicated and analyzed by SDS-polyacrylamide slab gel electrophoresis (Fig. 7, *inset*). Under nonreducing conditions the trypsin-activated, SDS-treated and gel-filtered C5 exhibited the same electrophoretic mobility and molecular weight as native, undigested C5 (Fig. 7 *inset*, left panel). Analysis under reducing conditions indicated that trypsin-activated, SDS-treated and gel-filtered C5 was predominantly in the C5' form since 80 ± 5% of the C5 α -chain fragments (on a molar basis) were present as C5 α_1 and C5 α_5 fragments that were disulfide bonded to the C5' molecule (Fig. 7 *inset*, right panel). To verify that SDS-treated and gel-filtered

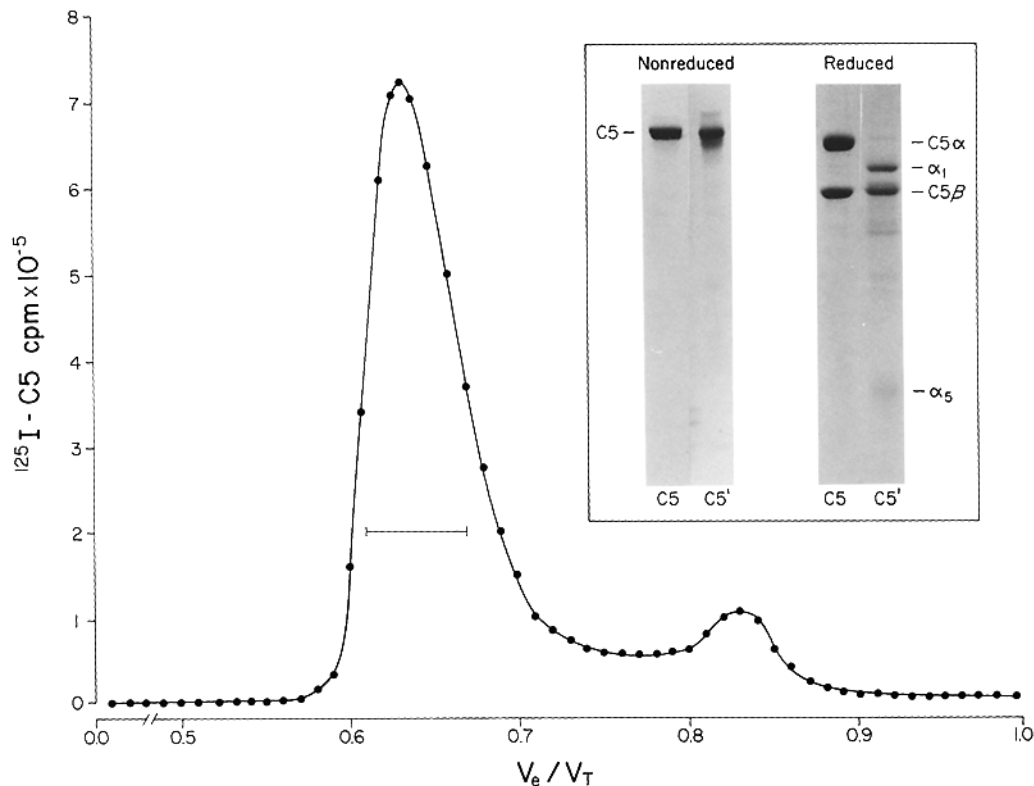


Fig. 7. Gel filtration column chromatography of trypsin-activated C5 in the presence of SDS. Human C5', prepared as described in the text, was preincubated with 2% SDS at 22°C for 60 min and subjected to gel filtration at a flow rate of 4 ml/h collecting 1-ml fractions at 22°C using a Sepharose CL-4B column (1.5 × 120 cm) equilibrated with PBS containing 0.2% SDS. The major peak fraction was pooled as indicated and subjected to SDS-polyacrylamide slab gel analysis in the presence (*reduced*) or absence (*nonreduced*) of β ME (*inset*).

C5' still retained C5a-like biological activity, the ^{125}I -C5' pool, as indicated in Fig. 7, was incubated at 2°C for 1 h to remove unbound SDS by precipitation. The C5' containing supernatant was dialyzed exhaustively against PBS, and assayed for C5a-like biological activity. As seen in Fig. 8, the SDS-treated and gel-filtered C5' still retained the ability to mediate the release of lysosomal enzymes from human neutrophils in a dose-dependent manner after SDS removal. This activity was shown to be a property of the C5' molecule and not of residual detergent because lactate dehydrogenase (LDH) activity was not present in the neutrophil supernatants (data not shown), and immunoaffinity-purified goat anti-human C5 antibodies completely blocked lysosomal enzyme-releasing activity (Fig. 8). In addition, the SDS was also removed, as described above, from the pooled fractions containing the small molecular weight fragments produced upon trypsin digestion of ^{125}I -C5 (Fig. 7), and assayed for PMN lysosomal enzyme-releasing activity. It was estimated that after SDS removal only 10% of the total recoverable C5a-like activity was expressed by the low molecular weight, C5-derived fragments, while 90% of the total recoverable C5a-like activity was expressed by the 210,000 molecular weight, C5' form of trypsin-activated C5 (data not shown).

C5 Activation by Various Noncomplement Enzymes. 5- μg portions of highly purified C5 were incubated with increasing concentrations of trypsin, α -thrombin, plasmin, kallikrein, or elastase (0.004–4 mol protease/mol C5) for 30 min at 37°C and subsequently analyzed for their ability to induce the release of *N*-acetyl- β -glucosaminidase from PMN lysosomes. As illustrated in Fig. 9, C5 digested with all the proteases except kallikrein, effected the release of PMN azurophilic lysosomal enzymes in a dose-dependent manner. Trypsin and elastase, under the incubation conditions employed, activated C5 to express biological activity at relatively low protease concentrations with as little as 0.004 mol trypsin and 0.01 mol elastase per mol C5 eliciting measurable quantities of activity. α -Thrombin at a concentration of 0.08 mol/mol C5 and plasmin at a concentration of 0.8 mol/mol C5 also exhibited the

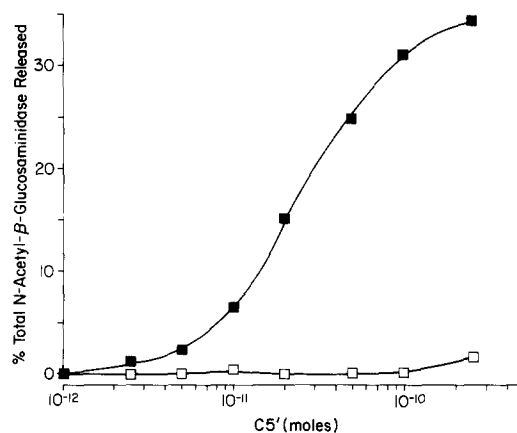


FIG. 8. Dose response of trypsin-activated C5' isolated in the presence of SDS. Human C5', which was SDS-treated, gel-filtered, pooled, and dialyzed as detailed in the text, was assayed for PMN lysosomal enzyme-releasing activity at the indicated concentrations. Identical samples were preincubated for 1 h at 22°C with a 50 molar excess of immunoabsorbent affinity-purified caprine anti-C5 antibodies (Materials and Methods) per mole C5' and monitored for remaining biological activity. ■, C5'. □, C5' + anti-C5.

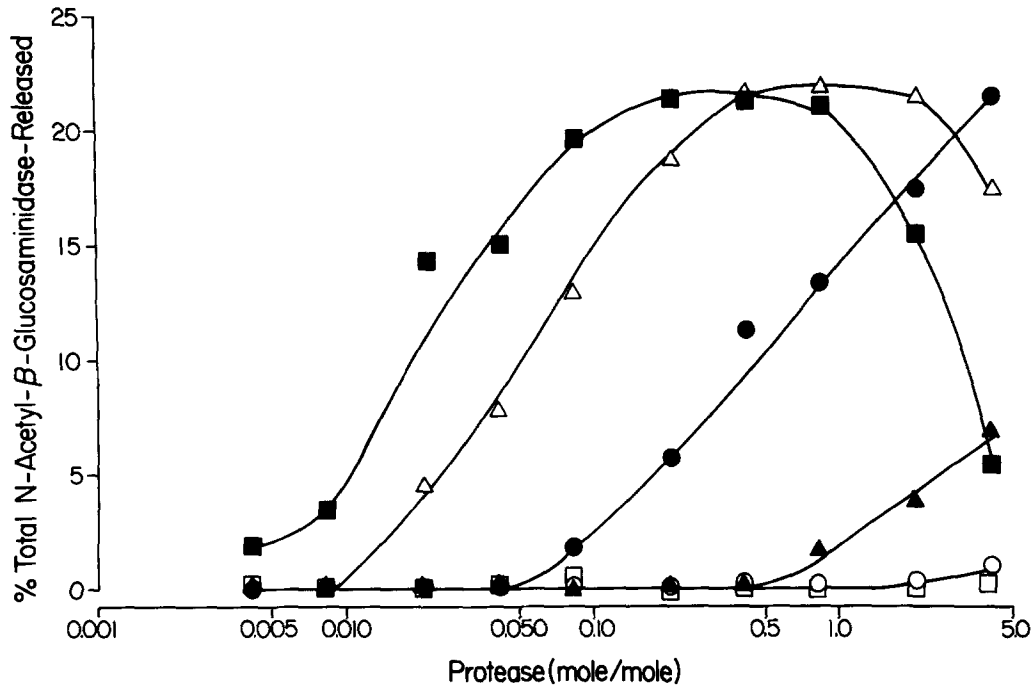


Fig. 9. Activation of C5 upon limited digestion with various proteases. Human C5 was incubated with the indicated molar ratios of trypsin, α -thrombin, plasmin, kallikrein or elastase, as detailed in the text, and assayed for the ability to induce the release of PMN lysosomal *N*-acetyl- β -glucosaminidase. ■, trypsin; ●, thrombin; ▲, plasmin; ○, kallikrein; △, elastase; □, LDH.

ability to generate enzyme-releasing activity from human C5. C5 alone, as well as the highest concentration of each protease employed alone, expressed no PMN lysosomal enzyme-releasing activity.

Site of C5 α -Chain Hydrolysis Mediated by α -Thrombin. Since previously published reports have indicated that α -thrombin can activate human C5 to express C5a-like biological activity (35, 50), it was of interest to determine the first site(s) of C5- α chain hydrolysis mediated by α -thrombin. Highly purified C5 was incubated with human α -thrombin (1 mol α -thrombin/mol C5) in 50 mM Hepes buffer, pH 7.5 containing 145 mM NaCl, 0.15 mM CaCl₂, and 0.5 mM MgCl₂ at 37°C for 20 h at a C5 concentration of 0.5 mg C5/ml. α -Thrombin digestion was stopped by the addition of PMSF to a final concentration of 2.5 mM, and the α -thrombin-treated C5 was subjected to SDS-polyacrylamide slab gel electrophoresis in the presence of β ME. The results from this study, presented in Fig. 10A, indicated that limited α -thrombin and trypsin digestion of C5 resulted in similar C5 fragmentation profiles. Namely, α -thrombin also cleaved the C5 α -subunit into C5 α ₁ and C5 α ₅ fragments (labeled C5 α ₁^{thr} and C5 α ₅^{thr} to distinguish them from the trypsin-produced fragments, C5 α ₁^{try} and C5 α ₅^{try}). Quantitative densitometric scans of these slab gel lanes (Fig. 10, B) further illustrated that the first site of α -thrombin and trypsin cleavage of the C5 α -chain were very similar. Furthermore, in analogy with trypsin activation, preliminary results have also suggested that the first α -thrombin-mediated fragmentation event that hydrolyzed the C5 α chain to α ₁^{thr} and α ₅^{thr} (C5'_{thr}) activated C5 to express C5a-like biological activity (50).

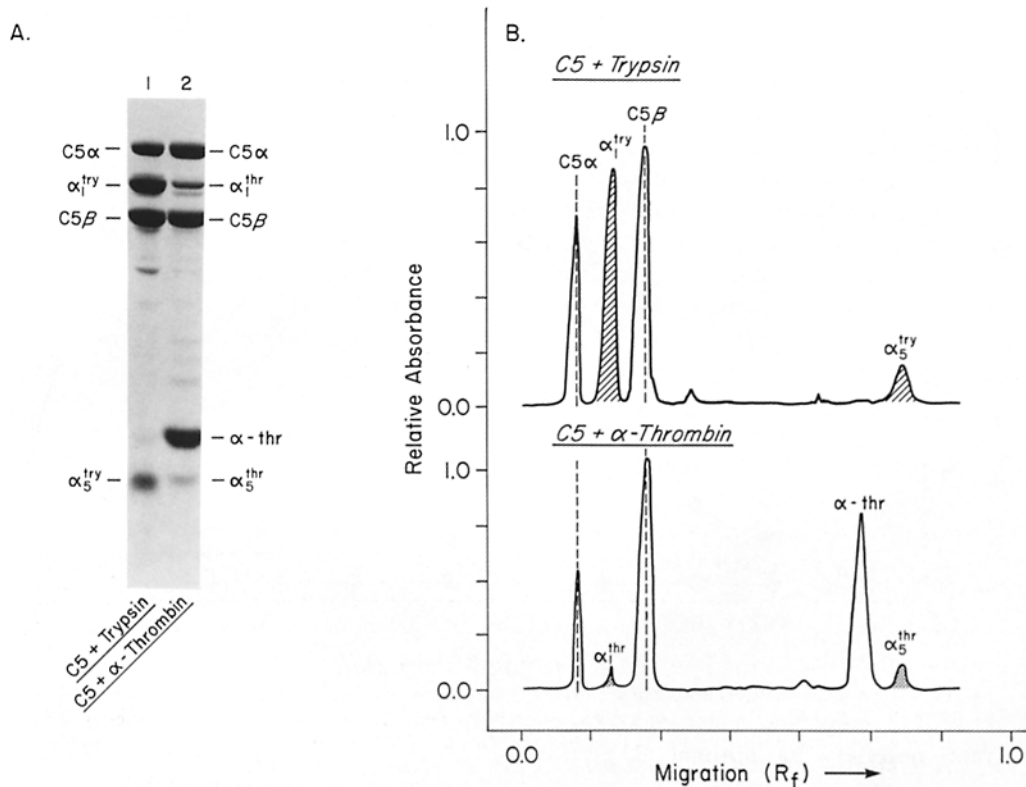


FIG. 10. Direct comparison between α -thrombin and trypsin produced C5 fragmentation profiles as examined by SDS-polyacrylamide slab gel electrophoresis. Human C5 was subjected to limited α -thrombin or trypsin digestion followed by SDS-PAGE analysis in the presence of β MME as outlined in the text (A). Densitometric scans were performed on the Coomassie Blue-stained gels and are presented in B. The densitometric scans were oriented with respect to C5 α - and β -chain mobilities.

Discussion

Several experimental variables were examined in an effort to maximize the ability of trypsin to activate human C5 to express C5a-like biological activities. When trypsin activation of C5 was conducted under optimal conditions, the trypsin-modified C5 mediated the directed chemotaxis and release of lysosomal enzymes from human neutrophils in a linear dose-response manner over a range of 0.1×10^{-12} to 0.3×10^{-12} mol per assay chamber and 1.0×10^{-12} to 3×10^{-12} mol per reaction mixture, respectively. In addition, our previously published results indicated that the molar concentration of trypsin-activated C5 required to attain maximal PMN chemotaxis (3×10^{-12} mol) and lysosomal enzyme-releasing activity (4×10^{-11} to 8×10^{-11} mol) was essentially identical to the concentration of highly purified C5a required to mediate peak responses in the same biological assay systems (25, 26). These results suggested that limited trypsin digestion of C5 resulted in the production and release of the C5a anaphylatoxin fragment. This presumptive conclusion that the primary or preferred site of trypsin hydrolysis of C5 resulted in the production and release of C5a or a C5a-related fragment(s) has been further supported by other previously published reports. Thus, Cochrane and Müller-Eberhard (15) clearly demonstrated that limited

trypsin digestion of human C5 resulted in the production of a 10,000 mol wt, C5-derived fragment upon gel filtration column chromatography at pH 4.0, that expressed C5a-like biological activity. Ward and Newman (21), utilizing sucrose density gradient ultracentrifugation, reported the production of an 8,500 molecular weight chemotactic factor generated upon trypsin digestion of C5, and Goldstein et al. (32) employing gel filtration column chromatography, showed that trypsin digestion of C5 yielded a low molecular weight fragment expressing neutrophil lysosomal enzyme-releasing activity. Therefore, the recently proposed hypothesis of Wetsel et al. (38), which suggested the principle mechanism by which trypsin activated C5 to express C5a-like biological activity does not require the release of a biologically active fragment(s), appears to be in direct conflict with these previously published results.

Nevertheless, the results presented in the present study can reconcile these apparently conflicting observations. Collectively, all of the currently available data clearly indicates that trypsin digestion of C5 can result in the production and release of a small molecular weight fragment(s), presumably C5a-related, which express a variety of phlogistic activities. However, under optimal activation conditions (0.008 mol trypsin/mol C5) <10% of the overall total C5a-like activity is represented by 5,000–15,000 molecular weight fragments (Figs. 4 and 7). Furthermore, the production of these 5,000–15,000 molecular weight fragments occurs only after extensive C5 α -chain digestion, and the quantity of fragments released cannot be increased even upon preincubation and gel filtration of trypsin-activated C5 in the presence of SDS (Fig. 7). The trypsin digestion conditions utilized by Ward and Newman (21) and Goldstein et al. (32) employed molar ratios of trypsin to C5 that were 100–400 times greater than necessary for optimal C5 activation (Figs. 1–3). Their digestion reactions therefore clearly utilized trypsin molar ratios in excess of any physiologically relevant C5 activation conditions attainable *in vivo*. Nevertheless, both studies (21, 38) did demonstrate the presence of uncharacterized, biologically active material approximating native C5 in molecular weight that exhibited C5a-like biological activity. However, this high molecular weight form of trypsin-activated C5 was not observed in the studies of Cochrane and Müller-Eberhard (15) since it was precipitated on the gel filtration column upon acidification (Fig. 6). Collectively, these previously published observations (21, 32, 38), in conjunction with the results presented in Figs. 3–5, and 7, indicate that the primary and preferred site of trypsin cleavage on the C5 α -chain results in the production of C5 α_1 and C5 α_5 fragments that remain covalently disulfide bonded to the modified C5 molecule (C5'). Furthermore, this primary trypsin-mediated cleavage event activates C5 to express C5a-like biological activity (Figs. 3 and 11).

Trypsin activation of complement proteins, particularly C3 and C5, may contribute significantly to the nonimmunologic inflammatory reactions observed in acute pancreatitis. Thus, the onset of acute pancreatitis is associated with a reduction in levels of total complement with the concomitant appearance of C3 fragments in the peripheral circulation (51, 52). In addition, Jacob et al. (53) have reported recently that the severe loss of vision, due to posterior retinal emboli of aggregated neutrophils, suffered by some patients with acute pancreatitis, was most likely mediated by trypsin-activated C5 since neutrophil aggregation induced by these patients' sera was inhibited by anti-C5 antibodies. In view of the present report, one would predict that a large proportion of the C5a-like biologically active factor present in the circulation

in the expression of C5a-like inflammatory reactivities. In fact, the data presented in Fig. 10 strongly suggests that α -thrombin, a centrally important enzyme in the coagulation system, activates C5 to express C5a-like biological activities by a mechanism that is virtually indistinguishable from that described for trypsin. Although the precise mechanism by which these noncomplement enzymes, other than trypsin, structurally modify C5 to mediate these reactions is currently unknown, the ability of these enzymes to activate C5 to express C5a-like reactivities should greatly broaden our concepts regarding the potential ability of C5 to act as a mediator of nonimmunological inflammatory reactions in a variety of physiological as well as pathological *in vivo* situations.

Summary

Experimental conditions required for the expression of maximum C5 activation upon limited trypsin hydrolysis were determined to be 0.008 mol of trypsin/mol C5 in a reaction mixture containing 1 mg C5/ml veronal-buffered saline incubated at 37°C for 30 min. Employing these optimal incubation conditions, the primary or preferred site of trypsin hydrolysis of the C5 α -chain resulted in the production of C5 α_1 (molecular weight, 90,000) and C5 α_5 (molecular weight, 25,000) fragments that remained disulfide bonded to the modified C5 molecule (C5'_{try}). Detailed structural-functional analyses clearly indicated the trypsin-mediated conversion of the C5 α -chain to C5 α_1 and C5 α_5 was responsible for the acquisition of neutrophil lysosomal enzyme-releasing and chemotactic activities. Gel filtration column chromatography under physiological ionic strength, pH 7.4, or in the presence of 0.2% SDS further demonstrated that at least 90% of the total recoverable C5a-like biological activity was mediated by the 210,000 molecular weight forms of trypsin-modified C5. Other physiologically relevant, noncomplement protease enzymes (α -thrombin, plasmin, and elastase) also activated C5 to express C5a-like reactivities. Analysis of α -thrombin-induced, C5 α -chain cleavage events by SDS-polyacrylamide slab gel electrophoresis indicated that the mechanism of α -thrombin-activation of C5 is similar to that described for trypsin. Reconciliation of this novel mechanism of C5 activation by trypsin with previously published results, and a discussion of the biological significance of noncomplement enzyme-mediated activation of C5 as it might relate to inflammatory processes *in vivo*, was presented.

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