FORMATION OF C3a AND C5a ANAPHYLATOXINS IN WHOLE HUMAN SERUM AFTER INHIBITION OF THE ANAPHYLATOXIN INACTIVATOR*‡

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Anaphylatoxins (ATs)[1] are low-molecular-weight reaction products of the complement system that have phlogogenic activity. They cause histamine release from mast cells, contraction of smooth muscles, and increased capillary permeability. Two distinct ATs have been described, one derived from the third (C3) and the other from the fifth (C5) components of complement. They are referred to as C3a and C5a, respectively (1).

The two peptides may readily be obtained from isolated human C3 and C5 (2–4). Paradoxically, their activities could never be demonstrated in whole human serum under conditions known to result in dissociation of C3a and C5a. Failure to produce the ATs in human serum had been interpreted in the past to indicate that they played no significant role in human biology. The demonstration of a highly efficient inactivator of the ATs in normal human serum (NHS) offered an alternate explanation (5). It was postulated that both peptides are liberated in human serum upon activation of the complement system, but that the expression of their biological activity is controlled by the AT inactivator (AI). Accordingly, it was anticipated that removal or inhibition of the inactivator would permit detection of both activities in whole human serum.

In the following it will be shown that C3a and C5a activity may indeed be produced in human serum from which AI activity has been abolished. A method will be described for the isolation of biologically active C5a from whole serum; and physical, chemical, and biologic properties of C5a will be compared with those of C3a.[2]

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[1] *Abbreviations used in this paper:* AI, anaphylatoxin inactivator; AT, anaphylatoxin; BSA, bovine serum albumin; C3PA, C3 proactivator; CPB, carboxypeptidase B; EACA, epsilon-aminocaproic acid; NHS, normal human serum.

[2] This material was presented in part at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology (6).

Materials and Methods

Serum.—Serum was obtained from fresh human blood, purchased from the San Diego Blood Bank.

Rabbit Antisera.—Highly purified preparations of AI and rabbit antisera to AI (5) and to human C3a (7) were obtained as described previously. Antiserum to human C5a was produced by injecting 40 µg of isolated C5a in complete Freund's adjuvant into the popliteal lymph nodes of rabbits. 3 wk later, 100 µg of the same material was injected subcutaneously and intramuscularly; injections were repeated weekly for 3 wk.

Immune Adsorption of AI with Insolubilized Anti-AI.—100 ml of rabbit antiserum to human AI was mixed with 100 ml of 0.2 M acetate buffer, pH 5.2; the protein was insolubilized by the addition of 16 ml of ethyl chloroformate. After thorough washing, the solid material (8) was suspended in 10 ml of NHS containing 0.05 M EDTA. The suspension was kept overnight at 4°C and then subjected to ultracentrifugation at 20,000 rpm for 1 h in a no. 40 rotor and a Spinco L2 centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The recovered supernatant was dialyzed against 2 × 10 liters of phosphate buffer, pH 7.0, ionic strength 0.05.

Elution of the Absorbed AI.—The immune adsorbent was washed with 2 × 50 ml phosphate buffer, pH 7.0, ionic strength 0.05, and once with 50 ml 1 M NaCl. The absorbed protein was then eluted with 3 × 50 ml of 4 M potassium iodide in 0.01 M Tris buffer, pH 8.2, and 0.1% bovine serum albumin (BSA) (9), allowing 15 min at room temperature for each elution. The eluted protein was concentrated in an Amicon concentration device (Amicon Corp., Lexington, Mass.) using an XM-50 ultrafilter.

Inhibition of AI in Human Serum.—The following amino acids and their analogues were tested: cysteine, arginine HCl, lysine-HCl, D-tryptophan-methyl ester-HCl, acetyl-L-lysine, hippuryl-L-lysine (Cyclo Chemical Div., Travenol Laboratories, Inc., Los Angeles, Calif.), epsilon-aminocaproic acid (EACA) (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), omega-aminocaprylic acid, pentenoic acid (Sigma Chemical Co., St. Louis, Mo.), and CdSO$_4$ (J. T. Baker Chemical Co., Phillipsburg, N. J.). Samples of NHS were incubated with different concentrations of these compounds for 3 h at 37°C or overnight at 4°C. Samples of treated serum ranging from 2 to 25 µl were tested for AI activity.

AT and AI Assays.—AT activity was assayed on segments of isolated guinea pig ileum as described previously (4). AI activity was detected by abolition of C3a or C5a activity after incubation for 2 min at 23°C and pH 7.5 (5).

Generation of C3a and C5a ATs in Human Serum.—C3a AT was generated by incubation, at 37°C for 60 min, of serum depleted of AI activity by immune adsorption or by inhibition with 1 M EACA. For the production of human C5a AT, AI-depleted serum was treated with 20 mg/ml of boiled (30 min) bakers' yeast at 37°C for 75 min. Standards of C3a and C5a ATs were produced by trypsinization of isolated C3 and C5, respectively (4, 7). Porcine AT was isolated by a modification of Vogt's method (1).

Isolation of C5a AT from Human Serum.—Usually 800 ml of fresh NHS was treated for 16 h at 4°C with 1 M EACA. The serum was then incubated at 37°C for 75 min with 20 mg/ml of boiled (30 min) bakers' yeast. An equal volume of cold distilled water was then added, and the pH was immediately lowered to 3.7 with 1 N HCl (approximately 800 ml). The reaction mixture was centrifuged twice at 10,000 rpm for 30 min to remove the yeast. The supernatant was then dialyzed against 2 × 10 liters of 0.1 M ammonium formate adjusted with HCl to pH 3.7.

Chromatography on Carboxymethyl (CM)-Cellulose.—The dialyzed sample was applied to a 3.5 × 50 cm column containing CM-cellulose that had been equilibrated with 0.1 M ammonium formate, pH 3.7. After application of the sample, the column was washed first with 4 liters of 0.1 M ammonium formate, pH 3.7, and then with 4 liters of 0.5 M acetic acid. The protein was eluted with 0.1 M ammonium formate adjusted with NaOH to pH 7.0. The frac-

tions containing AT activity that eluted between pH 5.5 and 6.5 were pooled, and the pool was concentrated to approximately 5 ml in an Amicon (Amicon Corp.) concentration device using a UM-2 ultrafilter.

Molecular Sieve Chromatography.—A 3.5 × 200 cm Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column was equilibrated with acetic acid-sodium acetate buffer, pH 3.7, ionic strength 0.1. A mixture of the sample and 10% (vol/vol) of a saturated sucrose solution was layered between the top of the Sephadex column and the supernatant buffer. The flow rate was 12 ml/h, and 3-ml fractions were collected. Fractions containing AT activity were pooled and concentrated.

Chromatography on CM-Sephadex C50.—The concentrated pools containing AT activity from two, and at times three, different gel filtrations were chromatographed, a 2 × 30 cm CM-Sephadex C50 column equilibrated with 0.02 M sodium acetate buffer, pH 5.5, being used. The protein was eluted with a linear salt concentration gradient from a conductance of 2.0-38 mmho/cm. 500 ml each of 0.02 and 0.5 M ammonium formate were adjusted with distilled water to a conductance of 2 and 38 mmho/cm, respectively.

Gel Filtration on Sephadex G-25.—A sample of the active material from the CM-Sephadex chromatography was labeled with ^{125}I (10). The specific radioactivity was approximately 4,500 cpm/μg of protein. Gel filtration was carried out on a 2 × 90 cm G-25 column equilibrated with acetic acid-sodium acetate buffer, pH 3.7, ionic strength 0.1. The flow rate was 4 ml/hr and 1-ml fractions were collected. The eluted protein emerged as a single peak after recycling.

Molecular Weight.—The molecular weight was estimated according to Andrews' method (11), employing a 3.5 × 200 cm Sephadex G-100 column equilibrated with acetic acid-sodium acetate buffer, pH 3.7, ionic strength 0.1. As reference substances, BSA, cytochrome c, and glucagon were used.

The polyacrylamide gel electrophoresis method of Hedrick and Smith (12) was also used for determinations of molecular weight. The gel concentration ranged from 5 to 12%. Beta-alanine acetic acid buffer, pH 4.5, was employed. Reference substances were lysozyme (14,500), papain in its monomeric and dimeric form (21,000 and 42,000), and trypsin (23,800).

Immunochemical and Electrophoretic Analyses.—The double diffusion in gel method was used for the detection of AI in NHS, in AI-depleted serum, and in the eluate from the anti-AI immune adsorbent. Monospecific rabbit antiserum to AI was used.

Immunoelectrophoresis was performed using 2% agarose in phosphate buffer, pH 6.0, ionic strength 0.05, with 0.01 M EDTA, and 2% agar in barbital buffer, pH 8.6, with 0.01 M EDTA. Disc electrophoresis was performed according to the method of Reisfeld et al. (13), 6% resolving gel and β-alanine acetic acid buffer, pH 4.5, being used. Analytical electrophoresis on cellulose acetate strips was carried out in a Beckman (Beckman Instruments, Inc., Fullerton, Calif.) microzone electrophoresis apparatus, model R-101, using a Beckman barbital buffer (pH 8.5), ionic strength 0.075, and 250 V for 20 min at 20°C.

RESULTS

Depletion of AI in Human Serum by Immune Adsorption.—Human serum was depleted of AI by immune adsorption. No AI was detectable in the treated serum by Ouchterlony test (Fig. 1). The protein eluted from the immune adsorbent gave a precipitin line identical with the line obtained with fresh NHS. The AI-depleted serum was no longer able to inactivate isolated C3a and C5a AT activity. The eluted protein that was immunochemically identical with AI lacked enzymatic AI activity. The activity was not restored by addition of magnesium chloride or cobalt chloride.
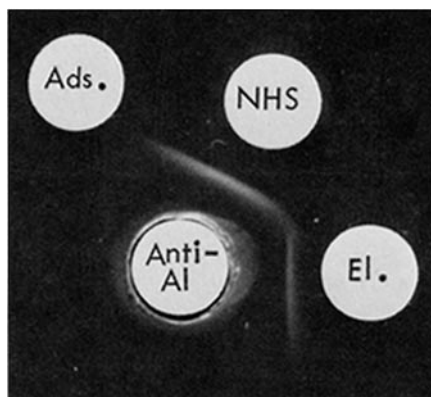
FIG. 1. Absence of immunochemically detectable AI in immune-adsorbed NHS (Ads.). Analysis was performed by Ouchterlony test using an anti-AI. The precipitating lines resulting from AI present in NHS (NHS) and the eluted protein from the solidified antibody (El.) fused completely.

AT Generation in Serum Depleted of AI by Immune Adsorption.—As depicted in Fig. 2, NHS by itself, after incubation at 37°C, or after activation with inulin, yeast, or immune complexes, was not able to generate smooth muscle contractile activity. However, after removal of AI, contraction of the guinea pig ileum was evoked by application of 50–100 μl of serum that had been incubated for 60 min at 37°C. The active material present in the serum was functionally identified as C3a since the muscle was unresponsive to 15 μg of isolated C3a. Further activation of the same serum with yeast caused full contraction of the C3a-desensitized guinea pig ileum. As we shall show, this material was identifiable with C5a AT.

Immunochemical Demonstration of C3a in Human Serum.—AI-depleted serum was incubated at 37°C for 60 min and then subjected to immunoelectrophoresis. The pattern was developed with anti-C3a. C3a was readily detectable as a cathodally migrating protein (Fig. 3). An antiserum to C3 revealed that about 50% of the protein had undergone conversion. Neither C3a nor the anodally migrating conversion products can be seen in untreated fresh serum. It is of interest that the conversion of C3 and dissociation of active C3a occurred at neutral pH.

Inhibition of AI in Whole Human Serum by Various Substances.—The compounds tested are listed in Table I. Cysteine and the chelators were the most effective inhibitors examined. Some amino acids or amino acid analogues were also active, but only at relatively high concentrations. Compounds with the amino group in the alpha, beta, and omega position were not inhibitory. Of all AI inhibitors, only EACA allowed generation of AT. All other inhibitors also inhibited AT formation. Inhibition of AI by EACA and cadmium sulfate is
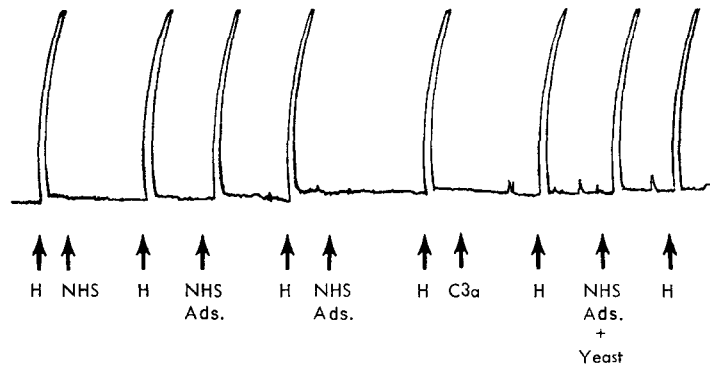
FIG. 2. Tracing produced by contraction of guinea pig ileum, showing biological activity in AI-depleted serum (NHS Ads.) after incubation at 37°C for 60 min without any additions. NHS, incubated NHS at 37°C for 60 min with immune complexes, inulin, or yeast. Repeated applications of NHS Ads. produced tachyphylaxis and cross-tachyphylaxis for isolated human C3a. However, the muscle was still fully reactive to histamine (H) and to the biological activity generated after incubation of NHS Ads. at 37°C for 60 min with yeast, which could be identified with C5a activity.
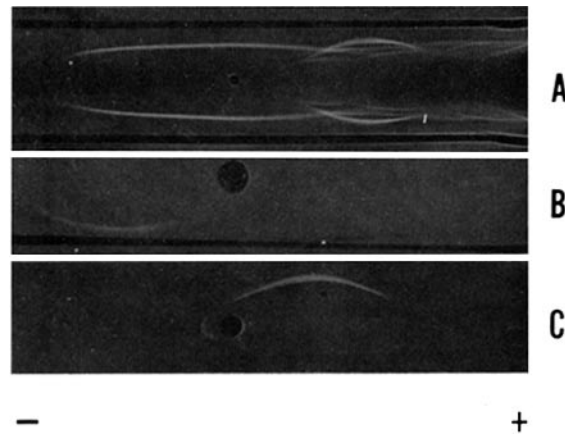

FIG. 3. Immunoelectrophoretic demonstration of C3a in whole human serum. (A) Untreated NHS, pattern developed with anti-NHS. (B) AI-depleted NHS incubated at 37°C for 20 min. Pattern was developed with anti-C3a, showing C3a near the cathodal end of the slide. (C) Untreated NHS developed with anti-C3a, detecting uncleaved C3.

similar to that of carboxypeptidase N or kinase I, described by Yang and Erdös (14). These substances do not inhibit kininase II.

Modes of AT Generation in Whole Human Serum.—Biologically active C3a and C5a may be obtained by treating whole human serum with a variety of substances after inhibition of AI by EACA. Thus, antigen-antibody complexes,

TABLE I

Examination of Various Compounds for Inhibitory Effect on Anaphylatoxin Inactivator

Compound	Concentration*	AI Inhibition (μl treated serum/10 μg C3a)		
		5	10	25
Cysteine	1×10^{-2}	+++	+++	+++
Arginine-HCl	2×10^{-1}	++	+	—
Lysine-HCl	7×10^{-1}	+++	++	±
Epsilon-aminocaproic acid	3×10^{-1}	+++	+	—
D-Tryptophan-methyl ester-HCl	1×10^{-1}	+++	+	—
1,10-Phenanthroline	1×10^{-1}	+++	+++	+++
EDTA	1×10^{-2}	+++	+++	+++
EDTA-Mg	1×10^{-2}	+++	+++	+++
CdSO$_4$	3×10^{-4}	+++	+++	+
S-2-aminoethyl-L-cysteine	5×10^{-1}	+++	++	—
Omega-aminocaprylic acid	5×10^{-1}	—	—	NT‡
Beta-aminopropionic acid	5×10^{-1}	—	—	NT
Alpha-aminocaproic acid	5×10^{-1}	—	—	NT
Pentenoic acid	1×10^{-1}	±	—	NT
Acetyl-L-lysine	5×10^{-1}	—	—	NT
Hippuryl-L-lysine	5×10^{-1}	—	—	NT

* The lowest effective concentration or the highest concentration tested.

‡ Not tested.

endotoxin, inulin, boiled bakers' yeast, and staphylococcal protein A were all effective. Treatment with trypsin also resulted in generation of AT, but in the appearance of kinin-like activity as well. For this reason, inulin or yeast were used as activators in all subsequent experiments.

Isolation of C5a AT from Human Serum.—Fresh NHS treated overnight with 1 M EACA was used as starting material. After activation with boiled yeast, the yeast was removed and the pH was adjusted to 3.7. This material was chromatographed on CM-cellulose in ammonium formate, and the AT activity was eluted between pH 5.5 and 6.5 (Fig. 4). After concentration, this material was passed over a column of Sephadex G-100. The elution profile and the position of AT activity in relation to known markers is illustrated in Fig. 5. Fractions containing AT activity from two, and at times three, different gel filtrations were pooled and chromatographed using a CM-Sephadex C50 column (Fig. 6). The peak of activity was eluted at a conductance of 32 mmho/cm and at a pH of 6.2. After concentration, the active material was passed over a Sephadex G-25 column and recycled to demonstrate molecular homogeneity of the isolated protein (Fig. 7). The purification achieved at each step of the isolation procedure is indicated in Table II. The average yield was 250 μg/liter of serum. Usually 17% of the original activity present in the activated serum was recovered. As will be described elsewhere, active C3a could also be isolated from whole human serum containing EACA.
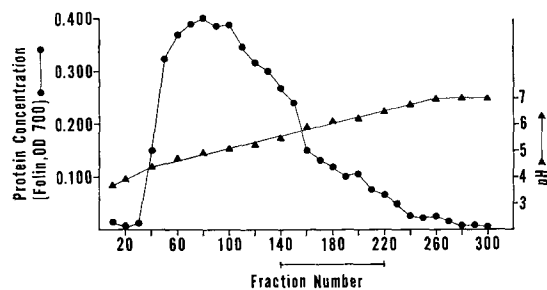
FIG. 4. First preparative step of C5a isolation procedure. Chromatography on CM-cellulose in a 3.5 × 50 cm column equilibrated with 0.1 M ammonium formate, pH 3.7. The portion of the chromatogram representing protein that was not absorbed is not shown. Fractions 140-220 were pooled and concentrated.
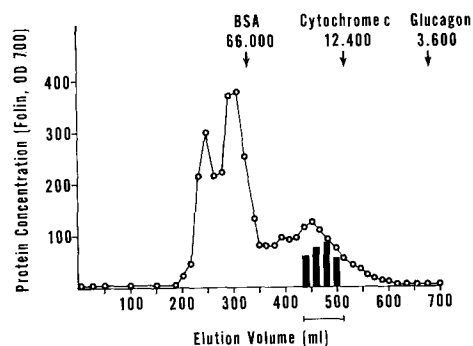

FIG. 5. Molecular sieve chromatography of C5a AT on a Sephadex G-100 column (2.5 × 200 cm) at pH 3.7. For comparison the elution volume of BSA, cytochrome c, and glucagon are indicated with their respective molecular weights.
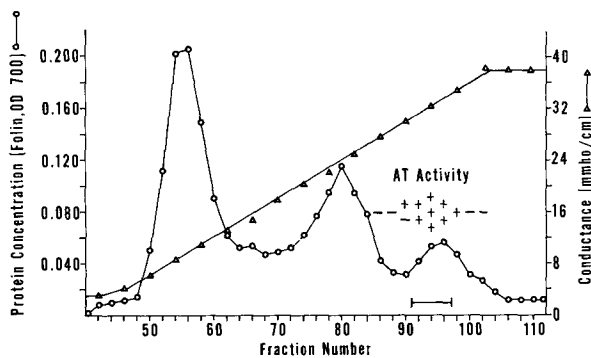

FIG. 6. Third step of C5a AT isolation: chromatography on CM-Sephadex C50. The protein was eluted with a gradient increasing in ionic strength and pH, from 2 to 38 mmho/cm and from pH 5.5 to 6.5, respectively (pH gradient is not shown). Active fractions were pooled and concentrated.
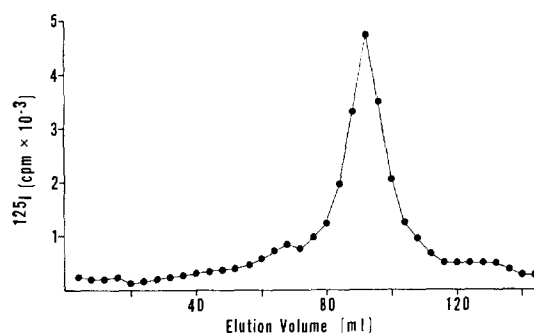
FIG. 7. Elution diagram of [^{125}I]C5a on a Sephadex G-25 column (2 × 90 cm) in acetic acid-sodium acetate buffer, pH 3.7, ionic strength 0.1. The diagram represents the second cycle on the same column.

TABLE II

Recovery and Yield of Human C5a

	Volume	Protein	Activity	Purification
	ml	*mg*	*mg*	
Serum	800	52,000	3.25	—
CM-cellulose	5	480	2.5×10^{-1}	1,300
Sephadex G-100	2	11	1.5×10^{-3}	2,150
CM-Sephadex C50	0.5	0.2	7.0×10^{-4}	4,600

Physicochemical and Biological Characterization of Human C5a AT.—The molecular homogeneity of the isolated protein was ascertained by physico-chemical and immunochemical studies. As shown in Fig. 7, gel filtration on Sephadex G-25 demonstrated a single major protein peak. The results of disc electrophoresis and immunoelectrophoretic analysis illustrated in Figs. 8 and 9 also showed a single major protein. The electrophoretic mobility of human C5a was studied by microzone electrophoresis and compared with that of pig AT (Fig. 10). By comparison with the known electrophoretic mobilities of albumin, transferrin, gamma globulin, and human C3a, the mobilities of human and pig ATs were estimated to be, respectively, -1.7 and -1.2 × 10^{-5} cm^2 V^{-1} s^{-1} (Fig. 11).

The diffusion coefficient was estimated by molecular sieve chromatography on Sephadex G-100 to be 12.1 × 10^{-7} cm^2/s and the molecular weight to be 17,500 daltons (Fig. 12). By the gel electrophoresis method a molecular weight of 16,500 was obtained (Fig. 13).

The minimal effective concentration when C5a AT was tested in the guinea pig ileum assay was 7.5 × 10^{-10} M. Introduced into the human skin by intra-dermal injection, the minimal effective dose required to produce a wheal and erythema reaction was 1 × 10^{-15} mol. As shown in Fig. 14, isolated AT ob-tained by contact activation of whole human serum produced completed
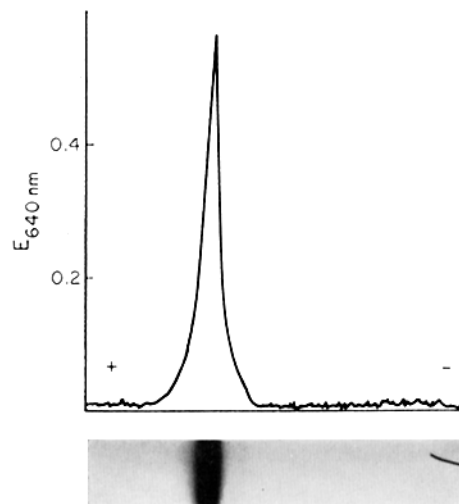
FIG. 8. Demonstration of C5a by acid polyacrylamide gel electrophoresis (6%) at pH 4.5. Top: extinction pattern of gel stained with amido black. Bottom: stained gel (75 μg of protein applied). Wire tip indicates position of buffer interface.
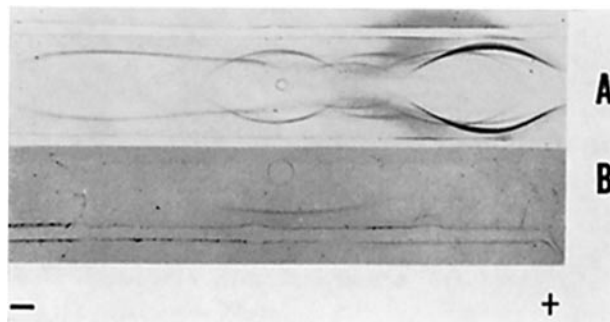

FIG. 9. Immunoelectrophoretic demonstration of C5a in 2% agar. (A) Immunoelectrophoretic pattern of NHS. (B) C5a, no precipitating line developed with anti-whole human serum. Bottom trough contained anti-C5a.

desensitization of the guinea pig ileum to C5a AT obtained by trypsin treatment of isolated C5. As indicated in the same figure, this AT was also inactivated by isolated AI. Smooth muscle-contracting activity was also eluted from polyacrylamide gels after electrophoresis of C5a, and the activity distribution coincided with the protein distribution.

DISCUSSION

Our present investigations demonstrated unequivocally the potential biological significance of both human ATs and of their control mechanism, the AI. We showed that removal or inactivation of AI allowed generation of both
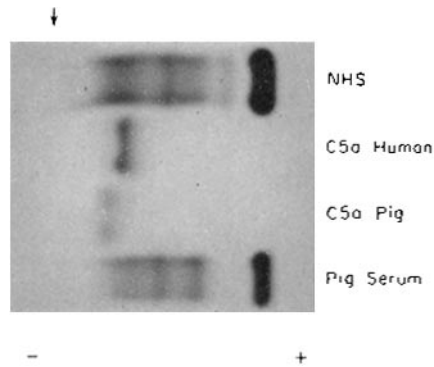
FIG. 10. Microzone electrophoresis at pH 8.5 of isolated human and porcine C5a. The arrow indicates the origin. For comparison the electrophoretic patterns of human and pig serum are shown.
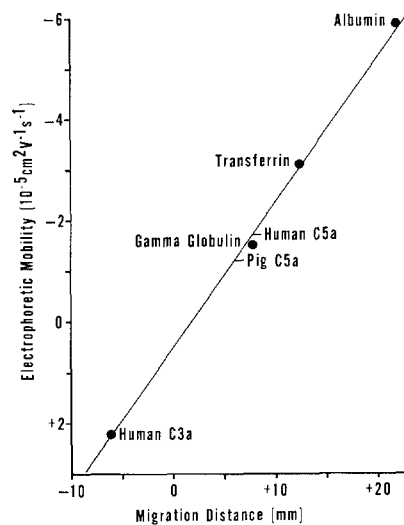

FIG. 11. Electrophoretic mobilities of human and porcine C5a ATs in relation to those of reference substances. The electrophoretic mobility was -1.7×10^{-5} cm^2 V^{-1} s^{-1} for the human and -1.2×10^{-5} cm^2 V^{-1} s^{-1} for the pig AT, respectively.

ATs, C3a and C5a, in whole human serum. Furthermore, both fragments could be demonstrated by immunochemical techniques. Thus, the well-known failure of human serum to serve as a source for ATs could be explained in terms of the function of the AI, the previously described carboxypeptidase B (CPB)-like enzyme of normal serum (5). Accordingly, human serum is not significantly different with respect to AT production from the sera of other species, such as pig, guinea pig, or rat.
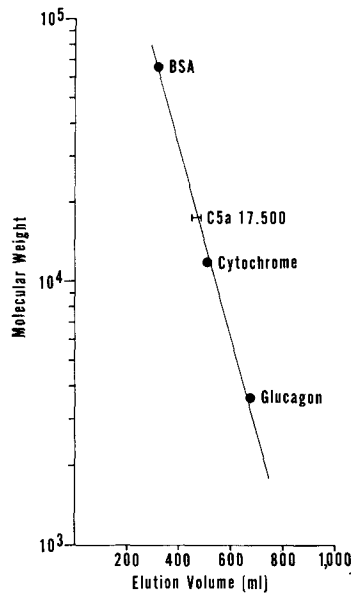
Fig. 12. Determination of molecular weight of human C5a by the method of Andrews, using a G-100 Sephadex column and reference substances. Molecular weight of C5a was found to be 17,500 daltons.
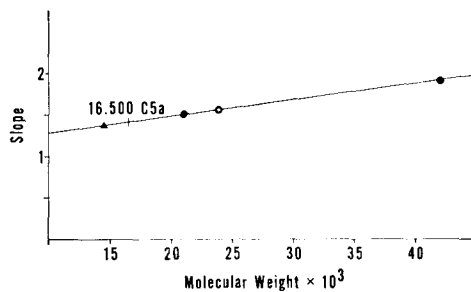

Fig. 13. Determination of molecular weight of human C5a by the gel electrophoresis method of Hedrick and Smith. The slopes were calculated from log relative mobility vs. gel concentration plots. Solid triangle, lysozyme (mol wt = 14,500). Solid circles, monomeric and dimeric form of papain (mol wt = 21,000 and 42,000, respectively). Open circle, trypsin (mol wt = 23,800). The molecular weight of C5a was found to be 16,500.

The difference between human and, for instance, porcine serum lies in the resistance of porcine C5a to inactivation by autologous AI. Further, a C3a analogue had never been demonstrated in whole serum of any species. As will be shown elsewhere,[3] C3a AT may be produced in whole porcine serum as it

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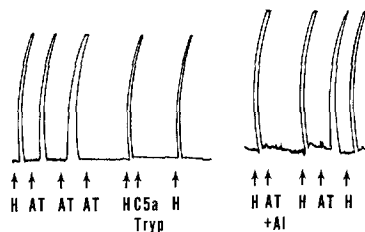[3] Vallota, E. H., T. E. Hugli, H. Ötting, and H. J. Müller-Eberhard. Manuscript in preparation.

FIG. 14. Biologic activity of C5a isolated from yeast-activated human serum. Tracing produced by guinea pig ileum contraction upon applications of 1 μg of C5a. Repeated application produced desensitization for C5a obtained by trypsin treatment of isolated human C5. Tracing on the right shows inactivation of human C5a by AI (AT + AI). After treatment of C5a with AI, no contraction could be elicited, whereas identical amounts of untreated C5a produced a full contraction (AT). H indicates the effect of histamine.

was shown in these experiments to arise in whole human serum. However, as in human serum, it is rapidly inactivated by the porcine AI. The differential susceptibility of porcine C3a and C5a to AI action results in suppression of C3a activity and accumulation of C5a activity. In contrast, both activities are suppressed in whole human serum. Formation and fate of human and porcine ATs are schematically represented in Fig. 15. The differential effect of AI on the ATs of experimental animals fully explains the fact that C3a escaped detection by the earlier students of AT.

Action of AI on C3a was shown to result in cleavage of the C-terminal arginine (5). Similarly, preliminary investigations of human C5a indicate that this peptide, too, has a basic residue in C-terminal position. Inactivation appears to be caused by removal of the C-terminal residue in both instances. Since AI also inactivates porcine C3a, its C terminus is most probably also occupied by a basic amino acid. The demonstration of a neutral residue, leucine, in C-terminal position for porcine C5a (15) is consistent with the resistance of the peptide to inactivation by AI. However, it raises an important question as to the identity and bond specificity of the C5a-liberating enzyme in porcine serum. Human C3a appears to be liberated by cleavage of a peptide bond involving the carboxyl group of arginine (16). The same appears to apply to human C5a. This is consistent with the tryptic nature of the C3- and C5-activating enzymes $\overline{C4,2}$ and $\overline{C4,2,3}$. The C3 activator of the alternate mechanism of complement activation also is trypsin-like (17), and this assumption may be made for the enzyme liberating porcine C3a. However, if porcine C5a is dissociated from the N-terminal portion of its precursor by cleavage of a leucyl bond, the bond specificity of the responsible enzyme would be different from that of its human counterpart. Such dissimilarity in enzyme specificity within the same biological system occurring in two mammalian species is improbable. The identity of the C-terminal residue of porcine C5a therefore requires reexamination.

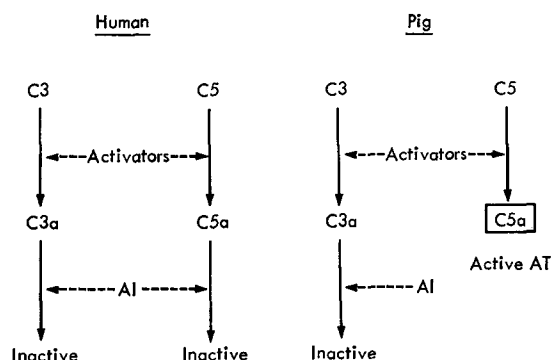Aging of NHS has been shown to result in slow conversion of C3 (18, 19).

FIG. 15. Schematic representation of current concept of formation and differential inactivation of ATs in human and porcine serum.

Our results indicated that after removal or inactivation of AI, mere incubation of NHS at 37°C resulted in generation of biologically and immunochemically detectable C3a. Addition of EACA accelerated the normally slow conversion to such an extent that no detectable native C3 was seen by immunoelectrophoresis after 4 h of incubation (Fig. 3). Whether EACA enhances a serum enzyme directly or indirectly involved in C3 conversion or whether it interferes with a serum enzyme inhibitor has not been determined.

Previously, human C5a was obtained by enzymatic cleavage of C5 with trypsin or the C4,2,3 enzyme (4). Our results indicate that C5a, as is the case for hog and rat ATs, can be generated by activation of whole serum with inulin or fresh or boiled bakers' yeast. These substances are known to trigger the alternate pathway. Generation of ATs under these conditions was always accompanied by conversion of the C3 proactivator (C3PA) to its enzymatically active form (20). Prevention of C3PA conversion by the use of high doses of heparin resulted in prevention of AT generation. Thus, AT formation by the methods used proceeded through the alternate mechanism of complement activation.

In the course of this work it was possible to demonstrate human C5a AT for the first time directly as a protein entity, through electrophoresis on acid polyacrylamide gels or cellulose acetate strips. The isolated peptide has a molecular weight of 16,500, which is more than twice that of C3a. It is not as basic as C3a; but when tested by intradermal injection in humans for causation of wheal and erythema, C5a appears almost 1,000 times more active than C3a on a molar basis (6).

Immunochemical and structural studies are under way to define the characteristics of isolated ATs from different species. For comparison, some of the properties of human C3a and C5a are listed in Table III. With the methods at hand for isolating both peptides in active form directly from whole serum, it has now become feasible to undertake comparative structural studies and to determine the chemical basis of the AT biologic activities.

TABLE III

Properties of Human C3a and C5a Anaphylatoxins

	C3a	C5a
Electrophoretic mobility*	+2.1	−1.7
Diffusion coefficient‡	—	12.1
Frictional ratio§	—	1.05
Molecular weight		
Gel filtration	8,700	17,500
Gel electrophoresis	7,200	16,500
Activity		
Minimal effective concn in vitro‖	2.5×10^{-8} M	7.5×10^{-10} M
Minimal effective dose in vivo¶	2.1×10^{-12} mol	1×10^{-15} mol

* Cellulose acetate, pH 8.5 ($\times 10^{-5}$ cm^2 V^{-1} s^{-1}).
‡ Sephadex gel filtration ($\times 10^{-7}$ cm^2 s^{-1}).
§ Calculated nomographically.
‖ Guinea pig ileum contraction.
¶ Wheal and erythema in human skin.

SUMMARY

Two biologically and chemically distinct anaphylatoxins (ATs) could be generated in whole human serum after removal of the AT inactivator (AI) by immune-absorption or after inhibition of AI with 1 M epsilon-aminocaproic acid (EACA). Both human ATs could be generated by treatment of serum with antigen-antibody complexes, which activate the classical complement pathway, and with inulin or yeast, both of which trigger the alternate pathway. The ATs were isolated from serum in active form and characterized as C3a and C5a.

Although human C3a had been characterized previously, C5a had not. The molecular weight of human C5a AT was 17,500; its electrophoretic mobility at pH 8.5 was $^{-}1.7 \times 10^{-5}$ cm^2 V^{-1} s^{-1}. The minimal effective concentration in vitro was 7.5×10^{-10} M. The minimal effective doses of human C5a in producing a wheal and erythema in the human skin was 1×10^{-15} mol. The results strongly suggest a biological function for both ATs and indicate that the expression of their activity is controlled by the AI of normal blood plasma.

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