Purification and Partial Characterization of Human and Porcine C3a Anaphylatoxin*

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SUMMARY

C3a anaphylatoxin is a protein fragment generated enzymatically in serum during activation of the third component of complement (C3). A four-step procedure is described for the purification of human and porcine C3a anaphylatoxins from their respective sera after activation with inulin. Because serum carboxypeptidase rapidly inactivates C3a, the inhibitor e-aminocaproic acid (EACA) was added during C3 activation, thus permitting isolation of fully active C3a anaphylatoxins directly from serum. A 2000-fold purification of C3a was achieved with an average 30% recovery assuming total conversion of C3 during treatment of serum with inulin. Human C3a anaphylatoxin obtained through the action of the C3 activating enzyme of the "alternate" pathway appeared nearly identical with the C3a obtained from isolated C3 after treatment with the C4,2 enzyme of the "classical" pathway or with trypsin.

Comparisons were made between various properties of human and porcine C3a anaphylatoxins. The molecular weights differed only slightly. Electrophoresis on a cellulose acetate strip at pH 8.6 indicated a difference of approximately one net charge between human and porcine C3a, with the human anaphylatoxin exhibiting the more basic behavior. Although the amino acid compositions are similar, significant differences exist. The most marked difference was the total absence of threonine residues in porcine C3a. The NH₂-terminal sequences of 20 amino acid residues were examined; homology existed for 16 of the 20 positions. Although partial analysis of the primary structure of human and porcine C3a indicates approximately 80% homology, no immunological cross-reactivity between the anaphylatoxins could be detected with antisera produced to either human or porcine C3a. In spite of the structural differences, the biological activities of porcine and human C3a were essentially identical. Smooth muscle contraction, increase in vascular permeability, and release of histamine from mast cells were similarly induced by equal amounts of anaphylatoxin from either human or porcine origin.

Porcine C3a, like human C3a, was shown to contain a COOH-terminal arginyl residue essential for smooth muscle contraction and for induction of histamine release from mast cells. The sequence adjacent to the COOH-terminal arginine was Leu-Ala-Arg-COOH for both human and porcine C3a. Current evidence suggests common mechanisms exist for the generation of C3a in various animal species and that the two known C3 activating enzymes in serum exhibit trypsin-like specificity.

C3a anaphylatoxin is a protein fragment released from the third complement component (C3) as a result of a selective proteolytic cleavage. Many biological activities have been attributed to anaphylatoxin, including the induction of histamine release from isolated mast cells (1, 2), increased vascular permeability (2, 3), intradermal wheal and erythema (4, 5), smooth muscle contraction (2, 6), and the chemotactic response of polymorphonuclear leukocytes (7-9). Two major pathways for complement activation, and hence for C3a generation, are known to exist in serum. The "classical" pathway involves the C4,2 enzyme (C3 convertase), which is formed by the action of C1 on the components C2 and C4 (10). A second distinct or "alternate" pathway involves complement activation by action of the enzymes of the C3 activator system (11, 12). A C3a-like anaphylatoxin also can be produced by action of trypsin or plasmin on isolated C3 (8).

Earlier investigations of the generation of C3a anaphylatoxin in serum have been impeded by the existence of a potent natural inactivator. This inactivator (13) is a serum carboxypeptidase which has the capacity to cleave the COOH-terminal arginyl residue of the C3a molecule. With the COOH-terminal arginyl

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residue removed, (C3ad is Arg), the C3a molecule loses its biological activities.

Anaphylatoxin inactivator (AI) exhibits a characteristic carboxypeptidase B-like specificity, since AI used hippuryl L-arginine and bradykinin as substrates as well as anaphylatoxin (13). In addition, action of AI on C3a in whole serum can be competitively inhibited by such known inhibitors of pancreatic carboxypeptidase B as EDTA, phenanthroline, arginine, or ε-aminocaproic acid. Of these agents, EACA has been successfully utilized in serum to inhibit the AI without interfering with the enzymatic processes necessary for generation of anaphylotoxins (14).

The following studies were undertaken in order to isolate fully active C3a anaphylatoxin directly from inulin-activated serum. Previous investigators have used enzymes from the "classical" pathway of complement activation for generating C3a from isolated C3 and the C3a produced in serum by enzymes from the "alternate" pathway of complement activation. In addition, detailed chemical analyses of C3a anaphylatoxins from human and porcine sources were performed for the purpose of correlating chemical structure with the biological functionality of anaphylatoxins.

**EXPERIMENTAL PROCEDURES**

*Materials*—Freshly drawn human blood was purchased from the San Diego Blood Bank. Fresh porcine blood was supplied by Natural Research Resources, Bonita, Cal. EACA was purchased from Schwarz-Mann. Inulin was obtained from Difco Laboratories. Small pore cellulose casing (23/32) was purchased from Union Carbide and prepared for use by boiling in sodium bicarbonate (1 g per liter) and EDTA (100 mg per liter) and washing several times in distilled water. Human C3a (0.5 mg) was radiolabeled with 2 mCi of 14C-arginine-T (10 μg), according to the method of McConahey and Dixon (16). Carboxypeptidase B (DFP-treated) was obtained from Worthington. Yeast carboxypeptidase Y (CpY) was a gift from Rikimaru Hayashi of Koyoto University, Koyoto, Japan. Sequenator grade chemicals were obtained from Beckman for use in the Beckman Automatic Sequencer.

**Anaphylatoxin Assays**—To assess the presence of anaphylatoxin activity, the ability of C3a to contract smooth muscle was routinely measured. Two-cm segments of guinea pig ileum were suspended in 10 ml of Tyrode's solution at 37°, and contractions were recorded according to the technique described by Cochran and Miller-Eberhard (6). Estimates of anaphylatoxin-induced histamine release from isolated rat mast cells were obtained according to the procedure of Johnson and Moran (17). Changes in the vascular permeability of guinea pig skin were detected by the method of Miles and Miles (18), using 0.5 ml of 2.5% Evans blue as the circulating dye.

**Cellulose Acetate and Gel Electrophoresis**—Electrophoresis of C3a on cellulose acetate strips was performed in a Beckman Microzone apparatus, model R 101. Beckman sodium diethyl carbonate (pH 8.6) with an ionic strength of 0.075 was used, and electrophoresis was carried out at 250 volts for 20 min at room temperature. Gel electrophoresis was performed in 12% polyacrylamide gels according to the procedure of Reisfeld et al. (19) with ε-aminonitroacetic acid at pH 4.5. Electrophoresis was accomplished at 8 to 10 volts per gel for 3 hours.

**Production of Antisera**—Antisera to human and porcine C3a anaphylatoxin were produced in rabbits by injection of approximately 100 μg of human or 200 μg of porcine C3a in complete Freund's adjuvant into the popliteal lymph nodes. A single booster injection of 40 to 50 μg of C3a was given intramuscularly 4 weeks after the initial injection, and the antisera were collected 1 week later.

**Analyses of the NH2- and COOH-terminal Residues of C3a**—A qualitative estimation of the NH2-terminal residues of human and porcine C3a was made by the dansyl chloride procedure of Gray and Hartley (20), and was used in conjunction with polyamide thin layer chromatography (21). Analyses of the COOH-terminal residues were made with a carboxypeptidase obtained from the fermenting yeast (22). Kinetic studies of amino acid release by 2% (w/v) carboxypeptidase Y were performed at 37° in 0.01 M sodium acetate, pH 5.5.

**Primary Sequence and Amino Acid Analyses**—Samples containing 1.5 to 3.0 mg of human or porcine C3a were reduced with 50 μl of mercaptoethanol and then alkylated with 100 mg of 1-14C-β-mercaptoacetate at pH 7.0 in 2 ml of 0.1 M sodium phosphate. The material was dialyzed against water and lyophilized. Alkylated C3a samples were dissolved in 0.5 ml of 0.1 M acetic acid, an aliquot was taken for protein determination, and the remainder was applied to the cup of an automatic sequencer.

A Beckman Sequencer, model 806B, was employed for automatic degradation of human and porcine C3a, using the direct Edman procedure (23). Samples corresponding to each residue step in the degradation process were converted to phenylthiolydantoin derivative of the amino acid by treatment for 1 hour in 1 N hydrochloric acid at 80°. Several analytical techniques were employed in the identification of amino acid residues. These techniques included gas chromatography of the PTI-derivatives, radioactivity measurements by liquid scintillation for identification of 14C-labeled carboxymethylcysteiny1 residues, and amino acid analysis of the residues released from PTI-derivatives following hydrolysis in HCl or 6 N HCl.

Amino acid analyses were performed with a Beckman amino acid analyzer (Spinco model 121) according to the methods described by Spackman et al. (24, 25). Protein samples were placed in Pyrex tubes containing 1 ml of constant boiling HCl, and the tubes were evacuated and sealed. Hydrolysis was performed at 110° for 24 hours, unless otherwise indicated.

**RESULTS**

**Anaphylatoxin Generation in Serum**—One liter of freshly drawn blood was clotted by incubation at 37° for 30 min. The clot was removed by centrifugation at 900 x g and 4° for 30 min. The serum was centrifuged again to remove residual red blood cells. A total volume of 400 to 500 ml of serum was obtained after centrifugation and solid EACA was immediately added to achieve a final concentration of 1 M. After standing at room temperature for 15 min, inulin was added (0.5 g/100 ml of serum). Optimal conditions for anaphylatoxin generation were found to be 1 hour of incubation at 37°. The reaction in serum was terminated by dropwise addition of glacial acetic acid (5 ml per 100 ml of serum), thereby adjusting the pH to approximately 5.5. Acidification assures inactivation of the serum carboxypeptidase (AI), which otherwise retains the potential of inactivating anaphylatoxin. The activated serum was centrifuged at 900 x g and 4° for 30 min to remove the suspended inulin.

**Purification of C3a Anaphylatoxin**—The initial step in the preparative procedure involved gel filtration of the activated and acidified serum. A Sephadex G-100 column (15 x 60 cm) was equilibrated with 0.15 M sodium acetate, pH 3.7. Samples large as 500 ml of activated serum were applied and the elution was performed at a rate of 200 to 250 ml per hour at 4° with the same 0.15 M sodium acetate buffer. In several of the preparations 14C-labeled human C3a was applied along with serum to

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3 A glass column was specially designed for large scale gel filtrations. The column consisted of a 3-liter Buchner funnel (coarse fritted disc) annealed to glass tubing (15 cm diameter), with a total height of 85 cm. The top of the column was tapered to 5 cm, permitting a direct connection to the reservoir line through a No. 11 rubber stopper fitting.
establish the elution profile of C3a. C3a was well separated from the majority of other proteins in serum; approximately 1.6 liters of the effluent were pooled, containing only 1% of the total protein applied. Following each use, the Sephadex G-100 column was stripped with 2 liters of 1 M sodium hydroxide to remove residual serum proteins and was again equilibrated with 0.15 M sodium acetate buffer.

The second step of the isolation procedure utilized CM-Sephadex to provide a means of concentrating and of further purifying the C3a. Approximately 600 to 800 ml of wet CM-Sephadex gel, previously equilibrated with 0.15 M sodium acetate at pH 3.7, were placed in a 3-liter coarse sintered glass Buchner funnel. The C3a pool from the G-100 gel filtration step was passed through the CM-Sephadex gel and the liquid was collected by gravity flow. This procedure was repeated by passing the recovered effluent over the gel a second time to assure complete adsorption of the C3a to the ion exchanger. The C3a molecule was found to adsorb so tightly that the gel could be washed with a liter of 1 M NaCl in 0.15 M sodium acetate, pH 3.7, without measurable loss of the material. High salt concentration was employed to cause concretion of the gel and displacement of loosely bound proteins. The CM Sephadex gel was transferred to a glass column (9 x 30 cm), and the absorbed protein was eluted with 0.1 M HCl. The C3a eluted along with the remaining protein in a total volume of 400 to 600 ml and these fractions were pooled and lyophilized. The material was carefully transferred from the lyophilizer flask with 30 to 40 ml of water and dialyzed in small pore dialysis tubing at 4 °C in 0.15 M sodium acetate at pH 3.7. Lyophilized protein did not fully dissolve until excess salt had been removed by dialysis.

A second gel filtration step was performed on a Sephadex G-100 column (4.5 x 70 cm) equilibrated and eluted with the same 0.15 M sodium acetate buffer. The dialyzed material from the CM-Sephadex column was applied and eluted at a flow rate of 30 to 40 ml per hour. It should be emphasized that a complete separation between the major protein contaminants and the anaphylatoxin was not obtained by this gel filtration step if the acidic effluent from the CM-Sephadex column was neutralized before lyophilization. It was presumed, therefore, that lyophilization under the employed acidic conditions leads to unfolding and aggregation of contaminant proteins, without adversely affecting anaphylatoxin structure or activity.

Fractions collected from the second G-100 gel filtration containing C3a activity were pooled and lyophilized. This lyophilized material was dissolved in 4 to 6 ml of water and dialyzed against 0.02 M sodium phosphate, pH 7.2, at 4 °C. The solution was applied to a DEAE-Sephadex column (2 x 20 cm) and eluted with 0.02 M sodium phosphate at pH 7.2. The C3a anaphylatoxin was recovered in the early fractions (Pool I) of the DEAE-Sephadex elution and was used without further purification. Identical isolation procedures were used for purification of porcine C3a.

Purity of C3a—The human and porcine anaphylatoxins obtained from the DEAE-Sephadex chromatography step were judged to be homogeneous by the following criteria. (a) The proteins migrated as single bands on polyacrylamide gel electrophoresis at pH 4.5. Electrophoresis of human and porcine C3a on cellulose acetate strips at pH 8.6 also gave single bands. (b) Using Ouchterlony double diffusion analysis and immunoelectrophoresis, rabbit antibodies made to purified human and porcine C3a gave single precipitin bands with human and porcine C3a, respectively. (c) Absence of the amino acid threonine in the total amino acid composition of porcine C3a anaphylatoxin (Table I) was taken to be an additional criterion of purity.

Some Properties of Human and Porcine C3a—Molecular weight determinations for human and porcine C3a were obtained by means of a calibrated Sephadex G-50 column. A molecular weight value of approximately 9000 was obtained from human and porcine C3a. Porcine C3a appears to be a slightly smaller molecule than its human counterpart, as judged by amino acid analysis (see below).

The immunochromatographic results indicated that human C3a did not give a detectable reaction with rabbit anti-porcine C3a antibody (Fig. 1A). Similarly, rabbit anti-human C3a did not react with porcine C3a, regardless of the relative proportions of antigen and antibody used. However, rabbit antibodies made to the purified human or porcine C3a molecules were capable of reacting with the corresponding precursor, i.e., native human or porcine C3 (Fig. 1B).

Amino Acid Compositions—Mean residue values for purified human and porcine C3a are presented in Columns I and III of Table I; a molecular weight of approximately 9000 is assumed for both proteins. For comparison, mean residue values for human C3a (see Column II, Table I) generated from isolated C3 by the action of C3 convertase (C4,2) are included (15). Mean residue values were taken from published data and were normalized to an assumed molecular weight of 9000. Since the similarity was striking and the variation minimal, significant differences probably do not exist between human C3a prepared by these two independent procedures. However, definite differences were observed between the amino acid compositions of human and porcine C3a. The most obvious differences were the complete absence of threonine and a lower content of arginine residues in porcine C3a as compared to human C3a. Net differences of at least 1 residue per C3a molecule also occurred for aspartic acid, glutamic acid, proline, alanine, and valine. Since the combined total of basic residues does not greatly outnumber acidic residues, a considerable amide content must exist to account for the very basic nature of both peptides.

COOH-terminal Analysis—Human and porcine C3a were rapidly inactivated by digestion with pancreatic carboxypeptidase B. Conversion of the active anaphylatoxin to an inactive form proceeds in minutes with 0.5% (w/w) carboxypeptidase B at pH 7 to 8. The electrophoretic behavior of the anaphylatoxins before and after carboxypeptidase B digestion were distinctive on cellulose. Amino acid analysis of the carboxypeptidase digests at pH 8.6 indicated 0.9 to 1.0 mol of arginine had been released per mol of the human or porcine C3a. Therefore, the respective differences in migration between native and carboxypeptidase B-treated C3a molecules on the cellulose acetate strip apparently represent a single net charge. In comparison, a difference of approximately one net charge apparently exists between the native human and porcine C3a molecules with the human C3a exhibiting greater basicity.

When human and porcine C3a were digested with yeast carboxypeptidase Y several amino acids were released in addition to the terminal arginine residue. A time course for carboxypeptidase Y digestion of both anaphylatoxins was performed. The results indicate that the COOH-terminal sequences for these two anaphylatoxins were identical for 3 residues: arginine, alanine, and leucine. However, the second anaphylatoxin was found to contain 4 residues present in the COOH-terminal sequence (15). Dansylation of both human and porcine C3a produced only the dansyl derivative of serine as the
Table I
Amino acid compositions of human and porcine C3a anaphylatoxin

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Human C3a</th>
<th>Human C3a (C42)</th>
<th>Porcine C3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysine</td>
<td>7.0 (7)</td>
<td>6.3</td>
<td>7.0 (7)</td>
</tr>
<tr>
<td>histidine</td>
<td>2.0 (2)</td>
<td>1.8</td>
<td>2.2 (2)</td>
</tr>
<tr>
<td>arginine</td>
<td>11.0 (11)</td>
<td>9.8</td>
<td>8.0 (6)</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>5.0 (5)</td>
<td>5.3</td>
<td>5.6 (6)</td>
</tr>
<tr>
<td>threonine</td>
<td>3.0 (3)</td>
<td>3.2</td>
<td>3.2 (0)</td>
</tr>
<tr>
<td>serine</td>
<td>4.0 (4)</td>
<td>4.0</td>
<td>3.8 (4)</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>8.5 (9)</td>
<td>8.3</td>
<td>10.9 (11)</td>
</tr>
<tr>
<td>proline</td>
<td>1.9 (2)</td>
<td>3.8</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>glycine</td>
<td>3.7 (4)</td>
<td>4.2</td>
<td>4.4 (4)</td>
</tr>
<tr>
<td>alanine</td>
<td>3.9 (4)</td>
<td>3.5</td>
<td>5.6 (6)</td>
</tr>
<tr>
<td>half-cystine</td>
<td>5.6 (6)</td>
<td>4.8</td>
<td>5.6 (6)</td>
</tr>
<tr>
<td>valine</td>
<td>3.2 (3)</td>
<td>2.9</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>methionine</td>
<td>2.8 (3)</td>
<td>2.5</td>
<td>2.8 (3)</td>
</tr>
<tr>
<td>isoleucine</td>
<td>2.0 (2)</td>
<td>1.8</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>leucine</td>
<td>6.7 (7)</td>
<td>6.9</td>
<td>6.9 (7)</td>
</tr>
<tr>
<td>tyrosine</td>
<td>1.7 (2)</td>
<td>1.6</td>
<td>1.7 (2)</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>3.1 (3)</td>
<td>2.6</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Total Residues | 77 | 75 | 76
Molecular Weight | 9,083 | 8,825

a Composition reported for human C3a released from isolated C3 by the action of C3 convertase (C42). Data taken from Budzko, Bokisch and Müller-Eberhard (15).

b Values corrected for destruction during acid hydrolysis according to Moore and Stein (26).

c Half-cystine residues were estimated both as S-carboxymethylcysteine and as cysteic acid.

d Tryptophan was determined in an independent sample of C3a after alkaline hydrolysis (27).

e ND, not determined.

FIG. 1. A, double immunodiffusion in gel analyses of human and porcine anaphylatoxins in agar. Rabbit anti-human C3a (Anti H) was added to the center well on the left side and rabbit anti-porcine C3a (Anti P) was added to the center well on the right side. One microliter of human C3a (1.2 mg per ml) and 1 μl of porcine C3a (1.1 mg per ml) were placed in the outer wells according to the labels H and P, respectively. B, immunoelectrophoresis of human and porcine C3a for 20 min in agarose gel at pH 6.0. The left slide shows a precipitin reaction with the anti-human C3a (Anti H) and both human C3a (H) and C3 in whole human serum (WHS). The right slide demonstrates an immunological reaction with anti-porcine C3a (Anti P) and both porcine C3a (P) and the C3 in whole porcine serum (WPS).
### Table II
Partial amino acid sequence of human and porcine C3a

#### Electrolytic sequence results after HI or HCl hydrolysis of the PTH-amino acid derivatives.

**Human C3a Anaphylatoxin**

<table>
<thead>
<tr>
<th>Position</th>
<th>Residue</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NH₂-Ser</td>
<td>HI: 26</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>HCl: 36</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Val-Glx</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Leu-Thr</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Glx-Lys</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Arg-Met</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Asx-Lys</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Val-Gly</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Tyr-Arg-</td>
<td></td>
</tr>
</tbody>
</table>

**Porcine C3a Anaphylatoxin**

<table>
<thead>
<tr>
<th>Position</th>
<th>Residue</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NH₂-Ser</td>
<td>HI: 26</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>HCl: 36</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Val-Glx</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Leu-Met</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Glx-Lys</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Arg-Met</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Asx-Lys</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Lys-Glx</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Tyr-Arg-</td>
<td></td>
</tr>
</tbody>
</table>

Assignments of amino acid residues were dependent on the background levels of contaminant amino acids remaining below 20%.

* Serine at position 1 in human and porcine C3a was qualitatively identified as the dansyl derivative according to the method of Gray and Hartley (20). A quantitative estimate of serine was obtained by measuring the breakdown product alanine after HI hydrolysis of the PTH-serine derivative.

* Glutamic and aspartic acids were not distinguished from the respective amides.

* Threonine was identified after HI hydrolysis as α-aminobutyric acid.

* Not determined.


#### Biological Activities and Stability

Several activities of human and porcine C3a were compared and the respective effects were found to be largely identical within the limits of the assays employed. Table III outlines the concentrations of the respective anaphylatoxins necessary to elicit a full contraction of the guinea pig ileum. The activities indicated here were comparable with those obtained for C3a isolated directly from purified C3 (8).

Both human and porcine C3a maintain full smooth muscle contracting activity after hours of exposure at room temperature to pH conditions of less than 1 or as high as 10, and after months of storage at 4°C between pH 3 and 7. Cross-desensitization of the ileum occurred between human and porcine C3a anaphylatoxins, but neither human nor porcine C3a caused desensitization of ileum for the C5a anaphylatoxin.

Histamine was released from isolated rat mast cells by human or porcine C3a, and the relative potency of these two peptides was essentially equivalent (see Table III). Extravasation of intravenously injected dye was measured to estimate the influence of C3a on vascular permeability. Significant areas of dye extravasation were observed in guinea pig skin on intradermal injection of 10⁻¹⁰ mol of either human or porcine C3a.

### DISCUSSION

Isolation of C3a anaphylatoxin directly from serum after activation of complement provides a new avenue for obtaining anaphylatoxin from human and animal sources. The procedure described above easily permits accumulation of quantities of C3a sufficient for carrying out extensive chemical and physical characteristics. Five hundred milliliters of human blood contains an average of 0.3 g of C3 (28) which, if completely converted, yields 15 mg of C3a anaphylatoxin. By the method described, 3 to 5 mg of C3a could be isolated from the same volume of blood representing an average recovery of 30% and a 2000-fold purification.

The mode of C3a generation employed in this study involved the enzymatic activation of C3 through the “alternate” pathway of complement activation (11, 12). The C42 enzyme of the “classical” pathway for C3 activation had previously been employed for generating C3a anaphylatoxin from isolated C3 (15). Additional routes have been described for generation of C3a-like anaphylatoxins from purified human C3, including limited tryptic digestion and chemical cleavage of C3 by treatment with hydroxylamine (29). C3a generated by the alternate pathway and C3a produced through the classical pathway were essentially identical in molecular weight, amino acid composition, and NH₂- and COOH-terminal amino acid residues. Anaphylatoxin generated by a limited tryptic digestion of C3 also exhibited a...
Fig. 2. Yields for amino acids released consecutively from human C3a anaphylatoxin by automatic sequence analysis. Two preparations of 120 and 270 nmol of carboxymethylcysteinyl C3a were analyzed in a Beckman Sequencer. The PTH-derivatives from one preparation (120 nmol) were hydrolyzed with HCl and those from the second preparation (270 nmol) with HI. Serine residues are detected as alanine (see text for assignment of a serine residue at position 1) and threonine residues are detected as a-aminobutyric acid after HI hydrolysis. Methionine residues were analyzed as methionine after HI hydrolysis (A) was performed at 110 ° for 24 hours and HI hydrolysis (B) at 120 ° for 18 hours. Recoveries of leucine at alternate steps in the sequence analysis represent typical levels of background amino acids (Δ).

![Graph showing amino acid yields](image)

TABLE III

<table>
<thead>
<tr>
<th>Response observed</th>
<th>Anaphylatoxin added</th>
<th>Human C3a</th>
<th>Porcine C3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle contraction*</td>
<td>1.1 to 1.4 \times 10^{-3} M</td>
<td>1.4 to 1.7 \times 10^{-3} M</td>
<td></td>
</tr>
<tr>
<td>Histamine release from mast cells*</td>
<td>4.4 \times 10^{-9} M</td>
<td>3.6 \times 10^{-9} M</td>
<td></td>
</tr>
<tr>
<td>Vascular permeability*</td>
<td>1.0 to 1.4 \times 10^{-10} M</td>
<td>1.0 to 1.4 \times 10^{-10} M</td>
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</tr>
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</table>

* Values represent concentrations of C3a required to produce full contractions of segments of guinea pig ileum suspended in 10 ml of Tyrode's solution at 37 °.

An arbitrary level of release was selected for making comparisons between human and porcine C3a. C3a concentrations represent amounts required to induce the release of 10 ng of histamine from 10⁴ rat mast cells. Rat mast cells were isolated by centrifugation through a 33% Ficoll solution according to Johnson and Moran (17).

Minimal doses of C3a which will produce a 2 to 4 mm area of blueing in guinea pig skin after the procedure described by Cochran and Müller-Eberhard (9). The data represent average responses from three animals.

Table III: Biological activities of human and porcine C3a anaphylatoxin

remarkable similarity to anaphylatoxin produced by complement activation.

Porcine C3a anaphylatoxin, which only recently has been demonstrated (30) and to date had never been isolated, was prepared by the same procedures described for the isolation of human C3a. Recovery of homogeneous C3a from porcine serum demonstrated the general applicability of the C3a isolation procedure for species other than human. The purification of porcine C3a provided an opportunity for comparing the chemical properties and biological activities of analogous anaphylatoxins from different species. Both human and porcine C3a exhibited identical biological behavior. The arginine at the COOH-terminal position of porcine C3a was essential for biological activity, because when this residue was removed enzymatically, an inactive C3blike derivative was formed. These results paralleled previous observations with human C3a (13). Other structural similarities between the two C3a molecules included identical sequences near the COOH termini, 6 half-cystine residues in each, an absence of tryptophan residues, and an identical content and distribution of hydrophobic and aromatic residues.

Finally, it should be emphasized that genetic polymorphism in human C3 is well documented. Multiple allotypes of human C3 are readily discerned by electrophoresis at pH 8.6 and have been grossly described as slow, intermediate, and fast electrophoretic forms (31, 32). Neither human nor porcine C3a exhibit any corresponding microheterogeneity (allotypy) when isolated from pooled serum. To date, partial NH₂-terminal sequence analyses of these two anaphylatoxins have indicated no ambiguities through 20 residues. Therefore, current evidence would suggest that the C3b portion of the C3 molecule retains the polymorphism detected by electrophoresis.

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