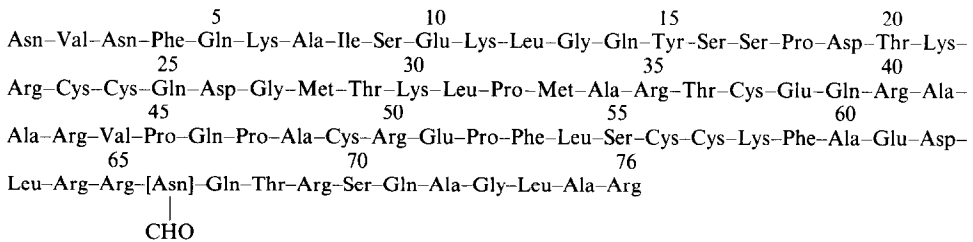# STRUCTURAL CHARACTERIZATION OF THE C4a ANAPHYLATOXIN FROM RAT

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*Institute of Basic Medical Science, Chinese Academy of Medical Sciences, Molecular Biology, 5 Dong Dan San Tiao, Beijing, China, †Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, MI 48109-0010, U.S.A. and ‡Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037, U.S.A.

Abstract—The C4a anaphylatoxin was purified from rat sera activated by heat-aggregated IgG. The anaphylatoxin was isolated by a three-step purification procedure and was judged to be homogeneous based on visualization of a single stained band after electrophoresis on both cellulose acetate membrane strips and on 9% SDS-polyacrylamide gels. Results from Ouchterlony and radioimmunoassay analysis indicated that neither rat C5A nor C3a contaminated the C4a preparation. Rat C4a is a glycoprotein estimated to be 11,000–12,000 mol. wt and contains 76 amino acid residues representing a mol. wt of 8577 and one oligosaccharide unit of 2000–3000 mol. wt. Rat C4a is weakly active in contracting guinea pig ileum at $0.1–1 \mu M$, which is comparable with the activity of human C4a. Both human and bovine C4a are polypeptides free of carbohydrate while rat and presumably mouse C4a are glycoproteins. The complete primary structure of rat C4a anaphylatoxin has been elucidated as follows:

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                5                    10                   15                  20
Asn–Val–Asn–Phe–Gln–Lys–Ala–Ile–Ser–Glu–Lys–Leu–Gly–Gln–Tyr–Ser–Ser–Pro–Asp–Thr–Lys–
            25                   30                   35                  40
Arg–Cys–Cys–Gln–Asp–Gly–Met–Thr–Lys–Leu–Pro–Met–Ala–Arg–Thr–Cys–Glu–Gln–Arg–Ala–
            45                   50                   55                  60
Ala–Arg–Val–Pro–Gln–Pro–Ala–Cys–Arg–Glu–Pro–Phe–Leu–Ser–Cys–Cys–Lys–Phe–Ala–Glu–Asp–
            65                   70                   76
Leu–Arg–Arg–[Asn]–Gln–Thr–Arg–Ser–Gln–Ala–Gly–Leu–Ala–Arg
                 |
                CHO
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INTRODUCTION

There are three complement derived anaphylatoxins, identified as C3a, C4a and C5a, and each display a variety of biological activities and have correspondingly distinct chemical characteristics. It has been shown that human C4a possesses weak spasmogenic activity both for inducing smooth muscle contraction and for enhancing vascular permeability (Gorski *et al.*, 1979). We have suggested that C4a functions as an anaphylatoxin and is genetically related to C3a and C5a (Hugli, 1984). Functional studies of synthetic peptides based on the COOH-terminal sequence of human and rat C4a provide perhaps the most convincing evidence that C4a is biologically related to the C3a anaphylatoxin (Hugli *et al.*, 1975). Analog synthetic pentapeptides based on the COOH-terminal sequence of human (AGLQR) and rat

(AGLAR) C4a were 0.05 and 17–25% as active, respectively, as was a pentapeptide LGLAR from human C3a in the ileal assay (Hugli *et al.*, 1983). Human C4a exhibits spasmogenic activity at levels of $10–20 \mu g/ml$ and based on the relative activity of synthetic peptide analogs rat C4a should be considerably more active than human C4a. On a molar basis, human C4a is less potent in all assays than are either C3a or C5a anaphylatoxins.

The primary structure of human C4a (Moon *et al.*, 1981) shows greater homology with C5a than with C3a. However, C4a is functionally related to C3a and not C5a based on cross-tachyphylaxis of the guinea pig ileum by C3a and C4a. Extending the studies of C4a to the rat system provides new insight into the relationships between structure and function of C4a. Furthermore, since C4a is generated specifically from activation of the classical complement pathway by antigen–antibody complex interaction with Cl, anti-rat C4a$_{\mathrm{des\,Arg}}$ antibody provides a useful reagent in developing immunoassays for detecting C4 conversion in this popular animal model. An immunoassay for rat C4a will prove useful in monitoring the course of immunologic diseases that involve the classical complement cascade in the experimental rat model.

MATERIALS AND METHODS

Bio-Gel P-60 and QAE Sephadex were obtained from Bio-Rad and Sigma Chemical Company, respectively. The Mono S HR 5/5 column was purchased from Pharmacia. Pooled rat serum was collected from two outbred strains (Wistar and Sprague–Dawley) of male and female animals by the Biotrol Company. The 2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid was obtained from Calbiochem, La Jolla. N-Glycanase™ (peptide: N-glycosidase F, peptide-N^4-[N-acetyl-beta-glucosaminyl] asparagine amidase) was purchased from Genzyme Corp., Boston, MA. All other reagents and solvents were of the highest chemical purity available.

Bioassay

Anaphylatoxin activity was assessed by measuring the contraction of terminal strips of guinea pig ileum (Cochrane and Müller-Eberhard, 1968).

Purification of rat C4a and C4a$_{des\,Arg}$

Two liters of rat serum was activated by adding 1 g of heat-aggregated (64°C for 60 min) human IgG in the presence of 2 mM serum carboxypeptidase N inhibitor, 2-mercaptomethyl-3-guanidino-ethylthio-propanoic acid (Hugli et al., 1982). The activated serum was acidified by slowly adding concentrated HCl to a final concentration of 1 N HCl and the precipitate was centrifuged. The supernatant, containing acid soluble proteins including the anaphylatoxins, was dialyzed against water in a small pore tubing then neutralized to pH 5–6 and lyophilized. Gel filtration of the lyophilized material redissolved in 400 ml of 0.05 M ammonium formate was performed at 4°C on a Bio Gel P-60 column (15 × 60 cm).

Elution from the P-60 column with 0.05 M ammonium formate at pH 5.0 was the first step of rat C4a purification. Initially the effluent was monitored by assaying for activity of the combined anaphylatoxins using the guinea pig ileal bioassay. Later the isolated C4a was radioiodinated with ^{125}I and elution was monitored as radioactivity (see Fig. 1). The fractions containing anaphylatoxin activity were pooled and fractionated further by applying the material to a QAE-Sephadex Q-50 column (1.8 × 20 cm), equilibrated with 0.05 M ammonium bicarbonate at pH 8.6 and developed using isocratic elution. This step removed a major portion of the contaminating protein (Fig. 2). The anaphylatoxin-containing pool was finally subjected to chromatography on a Mono S column. The conditions used to develop the Mono S column are described in the legend to Fig. 3.

Preparation of C4a$_{des\,Arg}$ can be obtained in the same manner as C4a except that the serum carboxypeptidase N inhibitor is not added. The C4a$_{des\,Arg}$-containing pools obtained from the Bio Gel P-60 and then a QAE-Sephadex column was applied to the Mono S column and the elution profile is shown in Fig. 4. These columns were all monitored by ^{125}I-C4a$_{des\,Arg}$ radioactivity and 280 nm absorbance. The material in the pool identified as peak I was C4a$_{des\,Arg}$.

The electrophoretic behavior of C4a and C4a$_{des\,Arg}$

Disc polyacrylamide gel electrophoresis was carried out on 0.1% SDS–9% polyacrylamide gels at pH 7.2 (Weber and Osborn, 1969) and protein bands were visualized by staining with Coomassie blue.

Electrophoresis on cellulose acetate strips was performed in 0.075 M barbital buffer at pH 8.6 using a Beckman Model R101 apparatus at room temp for 30 min at 200 V, and protein was stained using Amido Black.

Cyanogen bromide cleavage of C4a$_{des\,Arg}$

C4a$_{des\,Arg}$ was reduced and alklyated with 4-vinyl pyridine according to Friedman et al. (1970) prior to CNBr digestion. Pyridylethyl-C4a$_{des\,Arg}$ (PE-C4a$_{des\,Arg}$) was dissolved in 1 ml of 70% formic acid containing a 300-fold molar excess of CNBr. After incubation for 24 hr at room temp, the reaction mixture was diluted to 10 ml with cold water and lyophilized. The material was redissolved in 5 ml of water and again lyophilized. The dried material was dissolved in 0.2 ml of water and the CNBr-peptides were separated by reverse phase HPLC on a C4 column (Vydac 5). The elution conditions used are described in the legend to Fig. 6.

Amino acid and sequence analysis

Automated Edman degradation was performed on an Applied Biosystems Model 470A. The PTH amino acid derivatives were analyzed by HPLC using an automatic Waters HPLC system.

Amino acid analysis were performed on a Beckman 121M analyzer. Cysteine residues in C4a were reduced and alkylated by reaction with 4-vinyl pyridine before amino acid analysis was performed. The PE-C4a$_{des\,Arg}$ or CNBr-digested fragments of PE-C4a$_{des\,Arg}$ were placed in ignition tubes, vacuum sealed and hydrolyzed at 110°C for 24 hr in constant-boiling HCl containing 1% (v/v) phenol.

Determination of the C-terminal amino acid residues of C4a was accomplished by carboxypeptidase digestion. Rat C4a (2 nM) was dissolved in 1% sodium bicarbonate at pH 8.0–8.5 and incubated with 2% carboxypeptidase B (w/w) at 37°C. After 5 min, 0.1 M citric acid–0.2 M HCl buffer was added to stop the reaction. Amino acid analysis were performed to determine the residues released.

Carboxypeptidase Y digestion was performed in 0.05 M sodium acetate buffer at pH 5.5 and 37°C for 0–2 hr with 4% (w/w) enzyme. The reaction was stopped by adding 0.2 M citric acid buffer and amino acid analyses were performed.

Carbohydrate analysis

Aliquots containing 1.5 nmol of C4a$_{des\,Arg}$ were loaded on 0.1% SDS–9% polyacrylamide gels and disc electrophoresis was carried out at pH 7.2 (Weber and Osborn, 1969). One gel was stained with Coomassie blue for protein and another was fixed in methanol–water–acetic acid (60:35:5) and stained for carbohydrate by PAS reagents as described by Kapitany and Zebrowsky (1973).

Deglycosylation of native rat C4a was accomplished by digestion of 20 μg of rat C4a in 0.2 M sodium phosphate at pH 8.6 using 7.0 μl of N-glycanase (260 units/ml) and incubating for 12 hr at 37°C (Chu, 1986). The deglycosylated preparation of rat C4a was analyzed by SDS-PAGE as described above.

Immunoassay analysis

Monospecific anti-rat C4a was raised in rabbits by injecting 50 μg of rat C4a$_{des\,Arg}$ in complete Freund's adjuvant into the popliteal lymph nodes. Two and four weeks later the rabbits received intramuscular and subcutaneous injections of 50 μg of the antigen with incomplete Freund's adjuvant. One week later the rabbits were bled and antiserum was recovered. Immunodiffusion analyses (Ouchterlony and Nilsson, 1978) of isolated rat C4a, rat C3a (Jacobs et al., 1978) and rat C5a (Cui et al., 1985) were performed using the monospecific anti-rat C4a. Radioimmunoassays according to the procedures described by Hugli and Chenoweth (1981) were developed for rat C3a and rat C5a. Levels of both rat C3a and C5a were determined in rat C4a preparations using the radioimmunoassay technique at a sensitivity of 20–50 ng/ml.

RESULTS

Purification of rat C4a and C4a$_{des\,Arg}$

Both intact and des Arg forms of C4a were recovered from rat sera containing carboxypeptidase N inhibitor and activated by heat-aggregated human IgG. The results of a three-step procedure for purification of C4a are outlined in Figs 1–3. Gel filtration and QAE-Sephadex chromatography (Figs 1 and 2) result in a separation of rat C4a from rat C3a and most other impurities, but it fails to separate the C4a from C5a since both are cationic glycoproteins. Resolution of rat C4a and C5a was achieved by employing a Mono S column (Fig. 3). Results of amino acid analysis suggested that material in pools II and IV from the Mono S column was rat C4a and pools I and III presumably contain rat C4a$_{des\,Arg}$ (see Fig. 3). Although serum carboxypeptidase N inhibitor was present during complement activation with aggregated IgG, significant activation apparently occurred prior to the addition of IgG based on the level of C4a$_{des\,Arg}$ obtained. Presumably activation is caused both from mixing of serum obtained from several strains of animals and as a result of coagulation enzymes.
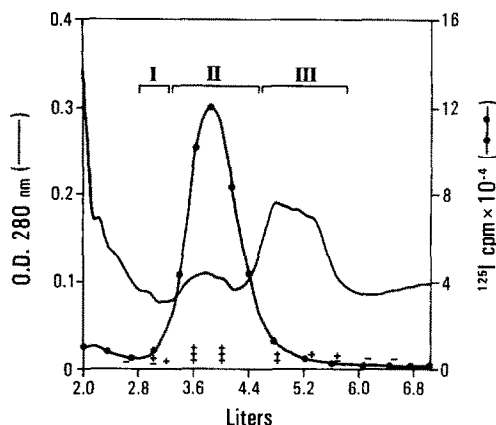

Fig. 1. Gel filtration of soluble material recovered from 2 l of complement-activated rat serum after acid precipitation. Separation was performed on a Bio-Gel P60 column (15 × 60 cm) equilibrated with 0.1 M ammonium formate buffer at pH 5.0. The column was eluted with 0.1 M ammonium formate, pH 5.5, at a flow rate of 135 ml/hr and fractions of 40 ml were collected. Biological activity was determined by the guinea pig ileal assay. Active fractions (35–80) coincided with radioactivity from [^{125}I]C4a$_{des\,Arg}$ that was introduced in the original serum sample. Material in pool II was recovered for chromatography on the OAE-Sephadex column.

Rat plasma drawn into EDTA contains 1.08 μg/ml of C4a and plasma drawn into heparin has 0.99 μg/ml of C4a which is 5–10-fold higher than normal background C4a levels in human EDTA or
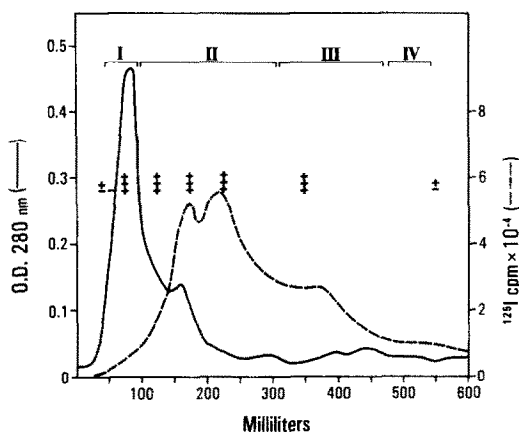

Fig. 2. Chromatography of material in pool II from the Bio Gel P-60 column was performed on a 1.8 × 20 cm QAE-Sephadex (Q-50) column equilibrated with 0.05 M ammonium bicarbonate at pH 8.6. Material in pool II collected from Bio Gel P-60 filtration was applied to the QAE-Sephadex column after dialysis, lyophilization and being redissolved in 1–2 ml of the pH 8.6 buffer. Isocratic elution was performed with 0.05 M ammonium bicarbonate at pH 8.6 at a flow rate of 10–12 ml/hr. Fractions of 2.5 ml were collected and biological activity was determined using the guinea pig ileal assay. Note that C4a and C5a activity was indistinguishable and overlapping at this step. The rat C3a elutes in pool I as the most cationic of the three anaphylatoxins (Jacobs et al., 1978).
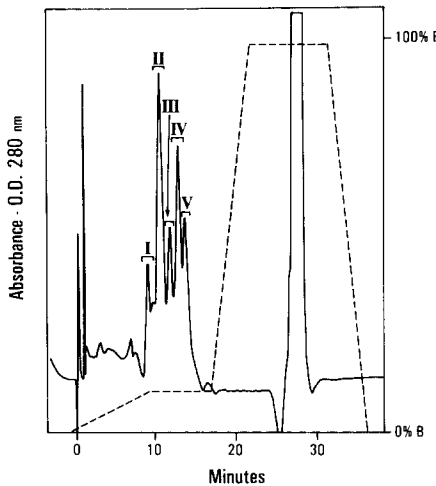
Fig. 3. Chromatography of C4a on a Mono S column. The anaphylatoxin-containing pools II and III from QAE-Sephadex were applied to a Mono S (HR 5/5) column. Solvent A: 0.1 *M* ammonium formate at pH 7.0. Solvent B: 0.8 *M* ammonium formate at pH 7.0. A linear gradient was developed over 10 min from 100% solvent A to 90% solvent A containing 10% solvent B. A 90% A/10% B solvent was maintained for 7 min and then another linear gradient from 10% solvent B to 100% solvent B was developed over 5 min. Elution with 100% solvent B was maintained for 8 additional min and returned to 100% solvent A. The absorbance setting was 0.02 O.D. units full scale. Both pools II and IV were concluded to contain C4a material based on amino acid analysis. We speculate that variations in sialic acid content may account for the multiple peaks of C4a.

Fig. 4. Chromatography of rat C4a$_{des\,Arg}$ on a Mono S column. Serum was activated by aggregated IgG without serum carboxypeptidase *N* inhibitor present and the anaphylatoxin-containing pool from Bio Gel P60 and QAE-Sephadex was applied to a Mono S (HR 5/5) column. Solvent A: 0.1 *M* ammonium formate at pH 7.0. Solvent B: 0.8 *M* ammonium formate at pH 7.0. A linear gradient was developed at 1 ml/min from 100% solvent A to 27% solvent B in 5.4 min and isocratic elution with 27% solvent B was maintained for 35 additional min. Pool I was determined to be rat C4a$_{des\,Arg}$.

heparin plasma. The C4a in rat serum was 7.63 ± 4.78 μg/ml (*n* = 48) or nearly half of the C4a expected from total conversion of C4. As in the plasma, we observed nearly 10-fold higher levels of C4a in fresh rat serum than in human serum. These results indicate that the predominant mechanism of C4 conversion during plasma or serum collection is initiated by enzymes of the coagulation cascade.

The major peak from a Mono S (Fig. 4) separation of material obtained by activating serum in the absence of carboxypeptidase *N* inhibitor contains the des Arg derivative of rat C4a. The materials in pool IV from Fig. 3 and in pool I from Fig. 4 are nearly identical based on compositional analysis except for the arginine content (see Table 1). When the material recovered from the Mono S column was applied to cellulose acetate strip electrophoresis (Fig. 5A), a single stained protein band was revealed indicating that intact C4a (lane 3) and C4a$_{des\,Arg}$ (lane 2) were homogeneous. Electrophoretic behavior of C4a on SDS-polyacrylamide gels (Fig. 5B) also shows a single band (gel 3) when stained with Coomassie blue. Positive staining of C4a in the SDS gels with PAS-reagent (gel 4) proved that rat C4a, like human C5a (gels 5 and 6), is a glycoprotein. Preparations of rat C4a and C4a$_{des\,Arg}$ obtained by the three-step isolation procedure were used in all the structural analysis and functional tests described below. Yields of approxi-
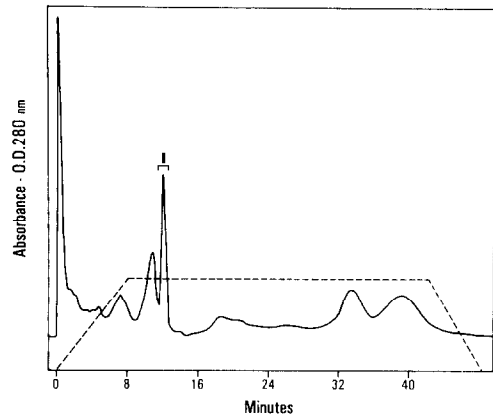
mately 1–2 mg each of C4a and C4a$_{des\,Arg}$ are recovered from 2 l of rat serum.

Anaphylatoxin activity of rat C4a

Both C4a and C4a$_{des\,Arg}$ were tested for anaphylatoxin activity. The results of the guinea pig ileal assay are given in Table 2 and only the intact C4a molecule is spasmogenic. Rat C4a$_{des\,Arg}$ is inactive at a concentration of up to $10^{-5}\,M$. Rat C4a elicits contraction of smooth muscle at a concentration of 0.1–1 μM, or approximately 3-fold greater activity than that of human C4a. Human C4a is active at a

Table 1. Amino acid composition of rat C4a and C4a$_{des\,Arg}$

Amino acid	C4a$_{des\,Arg}$ (residues/mole)	C4a[a] (residues/mole)
Lysine	4.90 (5)	4.89 (5)
Arginine	8.14 (8)	9.01 (9)
Aspartic acid	5.99 (6)	6.38 (6)
Threonine	3.75 (4)	3.68 (4)
Serine	4.26 (5)	4.51 (5)
Glutamic acid	11.23 (11)	11.05 (11)
Proline	5.57 (5)	5.54 (5)
Glycine	3.20 (3)	3.02 (3)
Alanine	8.15 (8)	7.86 (8)
Half-cystine	6.27 (6)[b]	6.0 (6)[c]
Valine	2.00 (2)	2.03 (2)
Methionine	2.05 (2)	1.40 (2)
Isoleucine	1.18 (1)	1.09 (1)
Leucine	5.26 (5)	5.12 (5)
Tyrosine	1.23 (1)	1.09 (1)
Phenylalanine	2.95 (3)	3.10 (3)
Total residues:	(75)[d]	(76)[d]

[a]Rat C4a was from pool IV of the Mono S column elution shown in Fig. 3 and rat C4a$_{des\,Arg}$ was from pool I of the Mono S column elution shown in Fig. 4.
[b]Half-cystine was detected as a pyridylethyl-cysteine derivative.
[c]Six half-cystines were assumed from C4a$_{des\,Arg}$ results.
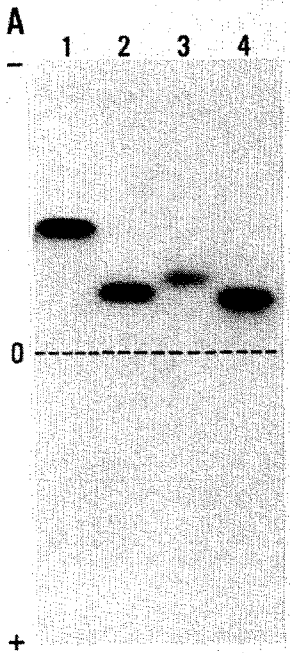[d]Residues in parentheses were taken from the primary structure analysis.

Fig. 5.(A). Microzone electrophoresis of rat C4a (lane 3) and rat C4a_{des Arg} (lane 2) compared with human C3a (lane 1) and human C5a (lane 4). Electrophoresis was performed for 20 min on cellulose acetate membrane strips at pH 8.6. The dashed line indicates the origin for sample application.
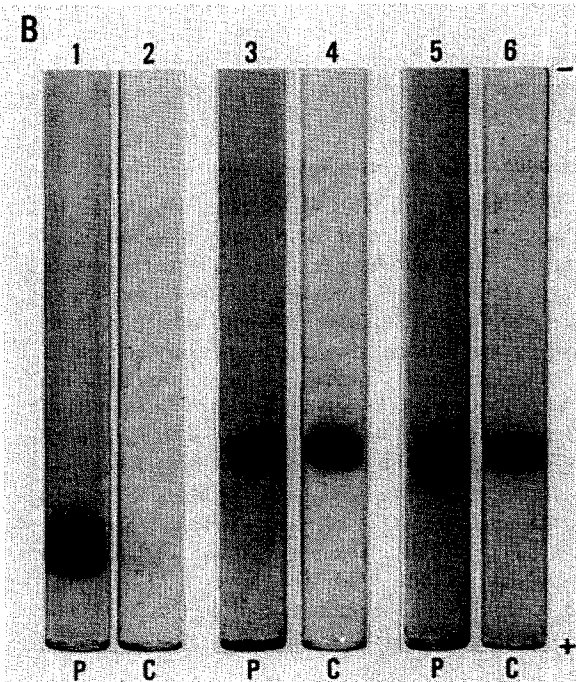

Fig. 5.(B). SDS-polyacrylamide gel electrophoresis of human C3a (gels 1 and 2), rat C4a (gels 3 and 4), human C5a (gels 5 and 6) in 9% acrylamide gels using 0.1% SDS. Protein was stained by Coomassie blue (left gel of pair) and carbohydrate (right gel of pair) was stained by PAS-reagent.

Table 2. Relative activity of rat C4a, human C4a and human C3a in the guinea pig assay

Factors	Effective doses[a]	Relative activity
Human C3a	$6\text{–}9 \times 10^{-9} M$	100
Human C4a	$1\text{–}2 \times 10^{-6} M$[b]	0.5
Rat C4a	$0.1\text{–}1 \times 10^{-6} M$	1.5
Deglycosylated rat C4a	$0.5\text{–}2 \times 10^{-6} M$	1.0

[a]Minimum concentration of factor required to elicit a full contraction of guinea pig ileal strip in a 2.0 ml bath.
[b]Cited from Gorski et al. (1979).

concentration of about 10–20 μg/ml (1–2 μM) while human C3a is active at a level of 0.05 μg/ml (5 nM).

Native C4a was readily deglycosylated by digestion with N-glycanase and without a requirement for detergent. When the deglycosylated rat C4a was electrophoresed either SDS-polyacrylamide gels or on cellulose acetate strips it migrated faster than intact rat C4a moving with rat C3a, which is a non-glycosylated anaphylatoxin. Deglycosylated rat C4a no longer stained positive with PAS staining and all of the glycosylated material was converted based on visual inspection of the protein staining pattern after electrophoresis. Functional analysis indicated that removal of the oligosaccharide unit from rat C4a had little influence on the spasmogenic activity of the molecule (see Table 2).

It is important to note that prior addition of human C3a to the muscle strips blocks rat C4a contraction, confirming that C3a and C4a are cross-tachyphylactic factors and are presumed to interact with the same cellular receptors. The fact that C3a blocks contraction of the muscle by C4a proves that the C4a preparation is devoid of rat C5a, since C5a-induced contractions are not affected by prior exposure to C3a. It is known that C5a is more than 1000 times as active as C4a on the guinea pig ileum, consequently trace quantities of C5a in the C4a preparation would be readily detected in the bioassay.

Amino acid sequence of rat C4a

Most of the structural analyses were performed using the des Arg form of rat C4a. The partial NH$_2$-terminal sequence of PE-C4a$_{des Arg}$ was established by automated sequence analysis and these results are presented in Table 3. This analysis of intact C4a$_{des Arg}$ provided assignments for 33 of the NH$_2$-terminal residues and identified two methionyl residues in C4a$_{des Arg}$ that are located at positions 28 and 33. From these results it was concluded that CNBr cleavage would produce a large peptide fragment containing residues 34–75, as well as the two sequenced fragments 1–28 and 29–33. The HPLC separation of a CNBr digest of PE-C4a$_{des Arg}$ was performed on a C4 reverse phase column (Vydac 5) which resolved the mixture into two major peaks (I and II) as shown in Fig. 6.

Amino acid analyses of the material recovered from pools CNBr I and CNBr II are shown in Table 4. The compositions of the two peptides are dis-

Table 3. The NH₂-terminal primary structure of rat PE-C4a_des Arg determined by automated sequence analysis

Cycle	Residue Identified	Recovery (pmole)
1	Asn	1920
2	Val	1860
3	Asn	1250
4	Phe	2050
5	Gln	1510
6	Lys	1000
7	Ala	1470
8	Ile	1110
9	Ser	100
10	Glu	640
11	Lys	490
12	Leu	700
13	Gly	270
14	Gln	400
15	Tyr	400
16	Ser	430
17	Ser	150
18	Pro	300
19	Asp	150
20	Thr	660
21	Lys	990
22	Arg	120
23	PE-Cys[b]	N.D.
24	PE-Cys[b]	N.D.
25	Gln	110
26	Asp	70
27	Gly	50
28	Met	80
29	Thr	40
30	Lys	30
31	Leu	80
32	Pro	120
33	Met	60

[a]Recovery was calculated from 1.32 nmole of rat PE-C4a_des Arg.
[b]PE-cysteine was positively identified but not quantitated.

Table 4. Amino acid analysis of peptides isolated from CNBr cleavage of rat PE-C4a_des Arg

Amino acid	CNBr-I (residues/mole)	CNBr-II (residues/mole)
Lysine	3.27 (3–4)[a]	1.13 (1)
Arginine	1.32 (1)	6.35 (7)
Aspartic acid	3.40 (4)	2.07 (2)
Threonine	1.46 (1–2)	1.86 (2)
Serine	2.20 (3)	1.72 (2)
Glutamic acid	4.62 (4)	7.13 (7)
Proline	2.04 (2)	2.93 (3)
Glycine	2.03 (2)	1.37 (1)
Alanine	1.69 (1)	6.74 (7)
Half-cystine	ND (2)[b]	ND (4)[b]
Valine	1.07 (1)	0.99 (1)
Methionine	0.14 (1–2)	0.05 (0)
Isoleucine	0.88 (1)	0.08 (0)
Leucine	2.04 (1–2)	3.14 (3)
Tyrosine	0.86 (1)	0.0 (0)
Phenylalanine	1.14 (1)	1.93 (2)
Total residues:	28–33[c]	42

[a]The number in parentheses were based on expected values from the primary structure analysis of rat C4a.
[b]Identified as PE-cysteine but not quantitated.
[c]Composition suggests mixture of fragments 1–28 and 1–33.

Short-term digestion of 2 nmol of C4a with carboxypeptidase-B (CpB) indicated that 1.07 mol of arginine was released per mole of C4a. Carboxypeptidase Y (CpY) digestion of 2 nmol of C4a_des Arg released the following residues expressed as moles per mole of C4a_des Arg: Ala 1.65, Leu 1.01, Gly 0.99 and Gln 0.47. These results gave further evidence that the COOH-terminal sequence of rat C4a is Gln–Ala–Gly–Leu–Ala–Arg.

Results from the partial NH₂-terminal sequence analysis of PE-C4a_des Arg and the CNBr fragment C4a 34–75, combined with the amino acid analyses of residues released from C4a by CpB and by CpY, provides a complete primary structure for rat C4a (Fig. 7).

Immunoassays for rat C4a

The rabbit anti-rat C4a detected only C4a by Ouchterlony immunodiffusion analyses and no cross-reactivity with purified rat C3a or C5a was observed. These results not only indicate that rat C4a is free of C3a and C5a, but suggest that these molecules share no common epitopes. Radioimmunoassay analysis is a more sensitive procedure than immunodiffusion and provides a quantitative indication of the levels of contamination in rat C4a by C3a or C5a. Radioimmunoassay for rat C3a and C5a were each negative when the pool of rat C4a form the Mono S column containing 1 mg/ml of rat C4a was tested. Since the levels of sensitivity detecting C3a and for C5a using this assay procedure is in the range of 20–50 ng/ml, we conclude that contamination of rat C4a by C3a or C5a is less than 0.002–0.005%.

tinctive and the composition of peptide from peak I clearly indicates that it represents residues 34–75 (peptide CNBr-II), a fragment from the COOH-terminal portion of C4a_des Arg. A sample of CNBr II was subjected to 42 cycles of automated Edman degradation and assignment of the sequence of the CN II peptide is given in Table 5.
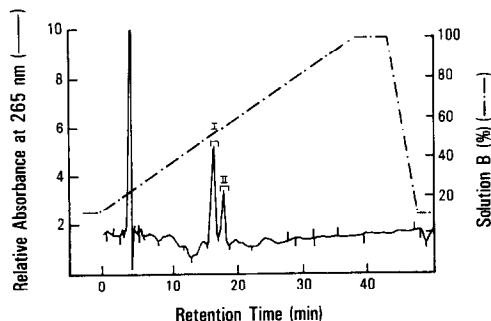

Fig. 6. Chromatography of the CNBr peptides derived from rat PE-C4a_des Arg on a Vydac 5 C4 column. Solvent A: 0.1% TFA in water. Solvent B: 0.03% TFA in acetonitrile. A linear gradient was programmed from 10% solvent B to 100% solvent B over 45 min at a flow rate of 1 ml/min. Peak I (contained fragments 34–75 of rat C4a_des Arg (peptide CNBr II) and Peak II contained fragments 1–28 and 1–33 based on amino acid analysis.

DISCUSSION

Rat C4a can be purified from rat serum activated by heat-aggregated IgG in the presence of a serum carboxypeptidase N inhibitor. However, a significant

Table 5. Automated sequence analysis of peptide CNBr-II from rat PE-C4a$_{des Arg}$

Cycles	Assigned residue position	Amino acid identified	Recovery[a] (pmole)
1	34	Ala	3630
2	35	Arg	3000
3	36	Thr	710
4	37	PE-Cys[b]	N.D.
5	38	Glu	1310
6	39	Gln	1660
7	40	Arg	320
8	41	Ala	1871
9	42	Ala	1730
10	43	Arg	460
11	44	Val	1620
12	45	Pro	1360
13	46	Gln	1020
14	47	Pro	1310
15	48	Ala	1530
16	49	PE-Cys[b]	N.D.
17	50	Arg	230
18	51	Glu	1050
19	52	Pro	730
20	53	Phe	2310
21	54	Leu	720
22	55	Ser	80
23	56	PE-Cys[b]	N.D.
24	57	PE-Cys[b]	N.D.
25	58	Lys	320
26	59	Phe	390
27	60	Ala	470
28	61	Glu	170
29	62	Asp	140
30	63	Leu	360
31	64	Arg	250
32	65	Arg	150
33	66	(X)[c]	N.D.
34	67	Gln	210
35	68	Thr	90
36	69	Arg	70
37	70	Ser	20
38	71	Gln	80
39	72	Ala	90
40	73	Gly	40
41	74	Leu	40
42	75	Ala	50

[a]Recovery was calculated based on 4.3 nmole of peptide CNBr-II.
[b]PE-cysteine was positively identified but recovery was not quantitated.
[c](X) indicates that residue was not identified.

quantity of rat C4a$_{des Arg}$ is also obtained from serum activated in the presence of the potent carboxypeptidase inhibitor. We presume that this C4 conversion occurs because serum collected from different strains of rats is mixed and heterologous blood group antigen–antibody complexes can activate C1. In addition, coagulation enzymes in rat blood contribute more to C4 conversion than in human blood based on relative C4a levels in plasma and serum. Yields of intact C4a, when carboxypeptidase inhibitor was added to rat serum (see Fig. 3) prior to activation by aggregated IgG, suggests that approximately half of the C4 escapes conversion by coagulation enzymes or from C1 activated by endogenous immune complex formation during blood processing.

Purity of rat C4a and the C4a$_{des Arg}$ derivative obtained by a three-step isolation procedure was examined by high resolution electrophoresis techniques. Electrophoretic separations of C4a (C4a$_{des Arg}$) by size or charge each indicated a single component. Sensitive radioimmunoassays that were specific for rat C3a or rat C5a detected neither factor in the C4a preparation. Based on the combined characterization results, the C4a fraction from the Mono S column was judged homogeneous. Functional and immunologic characterizations confirmed that rat C4a was free of C5a (<0.1%), a glycoprotein of similar size and charge.

Rat C4a is a glycoprotein with 76 amino acid residues and a molecular weight estimated from SDS-gels at 11,200, based on human C5a (Fernandez and Hugli, 1978). Determination of the complete amino acid sequence of rat C4a permits a comparison of this molecule with that of human C4a (Moon et al., 1981), bovine C4a (Smith et al., 1982) and mouse C4a (Nonaka et al., 1984). As shown in Fig. 8, the sequence of rat C4a indicates a 93.4% homology
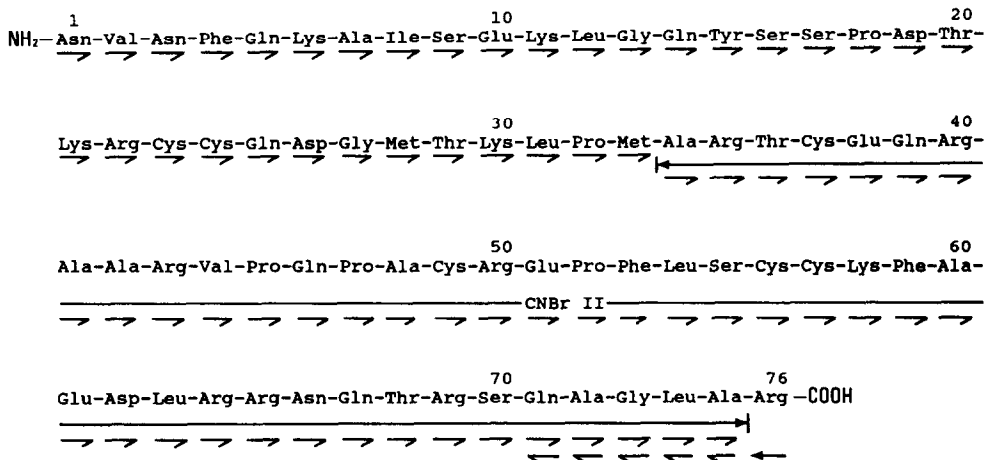

Fig. 7. The complete amino acid sequence for rat C4a. The N-terminal 33 residues were identified by automated sequence analysis of PE-C4a$_{des Arg}$. Residues 34–75 were identified by automated sequence analysis of CNBr II (———). Residues released by carboxypeptidase B (———) and Y (———) were designated by the arrows. Asn at position 66 was not identified but was assigned based on the amino acid composition of CNBr II and the assumption that this was the oligosaccharide attachment site in C4a.

```
              1                  10                  20                  30                  40
Rat   C4a    N  V  N  F  Q  K  A  I  S  E  K  L  G  Q  Y  S  S  P  D  T  K  R  C  C  Q  D  G  M  T  K  L  P  M  A  R  T  C  E  Q  R

Human C4a    -  -  -  -  -  -  -  -  N  -  -  -  -  -  -  -  A  -  -  T  A  -  -  -  -  -  -  -  V  -  R  -  -  -  M  -  S  -  -  -
Bovine C4a   -  -  -  -  -  -  -  -  H  -  -  -  -  -  -  -  T  -  -  V  A  -  -  -  -  -  -  -  -  L  -  R  -  -  -  -  -  -  -  -
Murine C4a   -  -  -  -  -  -  -  V  -  -  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  K  -  -  -  -  -  -
Murine Slp   -  -  -  -  L  -  -  L  -  -  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  K  -  -  -  -  -  -


                              50                   60          [CHO]           70                76
                                                                  |
Rat   C4a    A  A  R  V  P  Q  P  A  C  R  E  P  F  L  S  C  C  K  F  A  E  D  L  R  R  N     Q  T  R  S  Q  A  G  L  A  R

Human C4a    -  -  -  -  Q  -  -  N  -  -  -  -  -  -  -  -  -  -  Q  -  -  -  S  -  -  K  K  S  R  D  K  G  -  -  -  -  Q  -
Bovine C4a   -  -  -  -  Q  -  -  -  -  -  -  -  -  -  -  -  -  -  Q  -  -  -  S  -  -  K  K  A  R  -  -  G  -  V  -  -  -  -
Murine C4a   -  -  (G) -  -  Q  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  (A  I) -  -  -     -  -  -  -  -  -  H  -  -  -
Murine Slp   -  -  -  -  -  Q  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -     -  -  -  -  -  -  H  -  -  -
```

Fig. 8. Comparison of the rat C4a amino acid sequence with that of human C4a, bovine C4a, mouse C4a and mouse Slp. Dashes indicate homology with the rat C4a sequence. One deletion is positioned between residues 66 and 67 in both rat and mouse sequences compared with human and bovine C4a molecules based on maximal homology for neighboring residues. Note that rat C4a, mouse C4a and mouse Slp contain 76 residues while human and bovine C4a each contain 77 residues. The C5a and Slp sequences were deduced from cDNA clones of C4 obtained from B10.WR mice. Residues in parentheses identify differences in mouse C4a sequences deduced from a C4 cDNA clone obtained from another inbred strain of mice (FM).

between rat C4a and mouse C4a as deduced from the cDNA sequence of a clone from the B10.WR strain of mice (Sepich et al., 1985). Only five amino acid replacements occur between isolated rat C4a and the deduced sequence of mouse C4a from B10.WR ($C4^{W7}$) mice. Another deduced mouse C4 sequence was obtained from a Japanese inbred mouse strain FM (Nonaka et al., 1984) and the C4a region in $C4^M$ contains eight amino acid replacements (identified in parentheses in Fig. 8) compared with the rat C4a sequence. These results would indicate that the $C4^{W7}$ is more like wild-type than is $C4^{FM}$ based on the comparison with rat C4a.

A 75% homology exists between the rat and human C4a molecules, including six cysteinyl residues and a functionally essential COOH-terminal arginyl residue. A deletion of one residue exists in rat and mouse C4a when compared with the human C4a molecule. We have placed the deletion between positions 66 and 67 in the rat C4a based on maximal homology between flanking sequences. Comparison of the rat C4a sequence with human C5a indicates that an unassigned residue located at position 66 in rat C4a corresponds with the oligosaccharide binding site at position 63 in human C5a. It was therefore concluded that the glycosylation site near the C-terminus of rat C4a is analogous to that in human C5a. Furthermore, the deduced Asn–X–Thr sequence of residues 66–68 in rat C4a represents the only conventional oligosaccharide attachment site in the molecule. A similar glycosylation site exists in mouse C4a and mouse Slp; however it is unknown whether the mouse proteins are glycosylated.

Inactivity of rat C4a$_{des Arg}$ indicates that rat C4a, like all other C3a and C4a anaphylatoxins characterized to date, maintains a strict requirement of a C-terminal arginyl residue for biological activity. The biologic activity of rat C4a is somewhat greater than human C4a in the guinea pig ileal assay. Although

activity of a synthetic C-terminal analog peptide of rat C4a (e.g. AGLAR) is more similar to the human C3a pentapeptide LGLAR (Hugli et al., 1983) than it is to human C4a analog pentapeptide AGLQR, the spasmogenic properties of rat C4a compare more closely with human C4a than with C3a. The rat C4a C-terminal pentapeptide AGLAR is between 10 and 25% as active as human C3a LGLAR. Based on the fact that the rat C4a pentapeptide AGLAR is more than 100-fold more active as the human C4a peptide AGLQR, it remains inexplicable why rat C4a is so poorly active compared with human C3a. Obviously, the conformation of the C-terminal portion of C4a is as important for function as has been demonstrated for C3a (Lu et al., 1984). The fact that numerous residue substitutions occur near the functionally important C-terminus in rat, bovine and human C4a as compared to C3a may explain the unexpectedly low spasmogenic activity of C4a molecules.

We examined whether the carbohydrate moiety of rat C4a is somehow influencing expression of its biological activity. The composition of the carbohydrate moiety in rat C4a has yet to be determined; however the functional role appears not to be a major influence since enzymatic removal of the oligosaccharide unit resulted in no significant enhancement or reduction of C4a activity.

Since the rat is a popular experimental model for immune disease, the ability to monitor classical pathway activation in these animals may prove to be a valuable assay system. We and others have demonstrated that monitoring anaphylatoxin levels in human body fluids has been useful in correlating complement involvement with numerous disease processes (Chenoweth et al., 1981; Chenoweth, 1984; Hugli and Chenoweth, 1981). The rat antigen C4a should prove equally useful as an indicator of classical pathway activation in this animal model. Preliminary evidence suggests that the radioimmunoassay

methods of Gorski (1981) for quantitating human C4a in physiologic fluids may be adaptable for measuring C4 conversion in bodily fluids from the rat. Such measurements are valuable in assessing the role of complement in experimental models of immune disease.

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