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Primary structure and functional characterization of rat C5a: An anaphylatoxin with unusually high potency

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Abstract

The anaphylatoxin C5a is a pro-inflammatory factor generated from C5 during complement activation. C5a derived from rat C5 exhibits significantly greater potency compared to C5a from other species. Rat C5a was 25-fold more potent than human C5a for eliciting spasmogenic contraction of guinea pig ileum. Proteolytic removal of the C-terminal arginine of C5a ($C5a_{desArg}$) reduced spasmogenic potency of rat C5a by only 4-fold compared to a 3,000-fold reduction for human $C5a_{desArg}$. In addition, rat $C5a_{desArg}$ was 50-fold more potent than human $C5a_{desArg}$ in a guinea pig vascular permeability (in vivo) assay and as a chemotactic factor for human neutrophils. C5a and $C5a_{desArg}$ were purified from zymosan-activated rat serum. Rat C5a, like human C5a, is glycosylated but contains 77 amino acid residues instead of the 74 residues of human C5a. Comparison of the primary structures of rat and human C5a indicated differences at 30 positions including an insert of 3 residues (LLH) in the rat molecule between residue positions 3 and 4 in human C5a. Insertion of residues LLH between Gln-3 and Lys-4 in a recombinant human C5a molecule using site-directed mutagenesis failed to enhance potency. Synthetic C-terminal analogues of rat C5a proved to be measurably more potent than the corresponding human C5a analogues (Ember JA et al., 1993, *Protein Sci 2*(Suppl 1):159 [Abstr]). We conclude that multiple sequence differences in the C-terminal effector portion and/or elsewhere in rat C5a, but not the LLH insert, account for the significant enhancement in potency of rat C5a over C5a from other species.

Keywords: anaphylatoxin; complement; C5a; rat; spasmogen

As early as 1910, Friedberger demonstrated an activity in complement-activated animal sera that when administered intravenously was lethally toxic to guinea pigs (Friedberger, 1910). Because the treated animals appeared to undergo an anaphylactoid shock reaction, the term anaphylatoxin was coined to identify the lethal substance (Friedberger, 1910). Later it was reported that C5a generated in C-activated rat serum exhibited unusually potent in vivo effects in guinea pigs in comparison to C5a derived from other species (Friedberger et al., 1964). In guinea pigs, C5a administered intrabronchially can produce lethal spasmogenic effects that include long-term constriction of airways and the small arteries of the lung (Stimler et al., 1980). Collectively, these results warrant further investigation of the unique molecular properties of rat C5a that are responsible for higher toxicity and spasmogenic potency compared to C5a isolated from other species.

C5a is cleaved from the N-terminal end of the C5 α-chain by a specific C5 convertase (Hugli, 1986) during C activation (Hugli & Müller-Eberhard, 1978). Rat C5a is unusual in that the desArg derivative maintains a higher level of potency than does $C5a_{desArg}$ from other species (Hugli et al., 1987). Intact C5a exhibits a number of potent biological activities primarily associated with the inflammatory response. As a spasmogen, subnanomolar concentrations of C5a can induce contraction of smooth muscle tissue from a variety of animal sources (Hugli, 1978). C5a promotes histamine release from mast cells and basophils and can enhance vascular permeability (Johnson et al., 1975; Jose et al., 1981). Nanomolar concentrations of C5a also induce directed migration (i.e., chemotaxis) and degranulation

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Abbreviations: C, complement; C5a, proteolytic fragment of complement component C5; $C5a_{desArg}$, C5a lacking the C-terminal arginine; CM-Cys $C5a_{desArg}$, *S*-carboxymethylated-cysteine $C5a_{desArg}$; PE-Cys $C5a_{desArg}$, pyridyl ethyl-cysteine $C5a_{desArg}$; CpB or CpY, carboxypeptidase B or carboxypeptidase Y; FPLC, fast protein liquid chromatography; PAS, periodic acid Schiff; ED_{50}, C5a concentration required for half-maximal response.

of both leukocytes and monocytes (Hausman et al., 1972; Chenoweth & Hugli, 1980). The C-terminal arginine that is exposed when C5a is released from C5 is rapidly removed in vivo by a serum carboxypeptidase (serum carboxypeptidase N) (Gerard & Hugli, 1981) to produce $C5a_{desArg}$. Removal of arginine and concomitant reduction in C5a potency is presumed to be a control mechanism to inactivate C5a in vivo. However, it has been shown that arginine removal reduces C5a potency to a variable extent depending both on the animal source of C5a and the biological response or assay used for measurement of C5a potency (Gerard & Hugli, 1981; Gerard et al., 1981).

Elucidation of the primary structures of human, porcine, bovine, and mouse C5a (Fernandez & Hugli, 1976, 1978; Gerard & Hugli, 1979, 1980; Gennaro et al., 1986; Wetsel et al., 1987) indicate that these proteins share a high degree of similarity with respect to size (74 residues for human, porcine, and bovine and 77 residues in mouse) and primary structure (overall ≈70% identity). In addition, the tertiary structures of human and porcine C5a are highly similar, as determined by NMR (Zuiderweg et al., 1989; Williamson & Madison, 1990). C5a consists of 4 helical bundles arranged in an antiparallel orientation. The N-terminal and C-terminal regions are largely helical and are stabilized by hydrophobic side chain interactions with neighboring residues (Carney & Hugli, 1993). The internal "core" is stabilized by 3 disulfide linkages (Zuiderweg et al., 1989; Williamson & Madison, 1990).

Here we report elucidation of the primary structure and biological characterization of rat C5a. Rat C5a and $C5a_{desArg}$ were shown to be substantially more potent than human C5a and $C5a_{desArg}$ in a variety of biological assays. Structural characteristics of rat C5a and C5a molecules from other species are discussed in terms of structural differences that may account for the significantly higher potency of rat C5a.

Results

Chemical characterization of rat C5a ($C5a_{desArg}$)

Rat C5a and $C5a_{desArg}$ were obtained from zymosan-activated rat serum by a 3-step purification procedure as described in the Materials and methods (Fig. 1). To produce intact rat C5a, a carboxypeptidase inhibitor was added to the serum to prevent proteolytic removal of the C-terminal arginine by carboxypeptidase N during complement activation. The inhibitor DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid was used at 2 mM in preference to 1 M 6-aminohexanoic acid (Vallota, 1978) because 1 M 6-aminohexanoic acid (Hugli et al., 1981) interfered with the acid precipitation step in the isolation procedure. Microzone electrophoresis of rat C5a and $C5a_{desArg}$, obtained from the Mono-S column (see Fig. 1C), on cellulose acetate strips indicated a single protein band. Rat $C5a_{desArg}$ migrated to a more anodal position than did rat C5a as was expected (Fig. 2A). The SDS-polyacrylamide gel electrophoresis of rat C5a also indicated a single protein band whether stained for protein with Coomassie blue or for carbohydrate with PAS reagent (Fig. 2B). These results provided further evidence that rat C5a was homogeneous and, like human C5a (Gerard & Hugli, 1979), is a glycoprotein.

As presented in Table 1, the amino acid composition of rat C5a proved identical to that of rat $C5a_{desArg}$ except that rat C5a contains 1 additional arginyl residue. Rat C5a, like anaphyla-

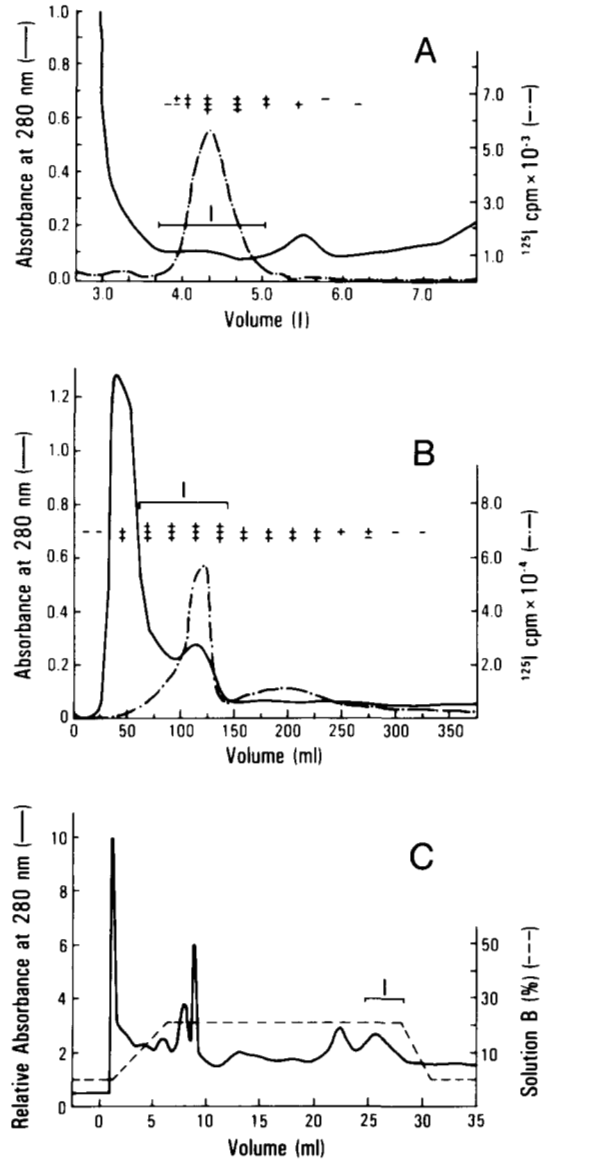

Fig. 1. A: Gel filtration profile for acidified supernatant from yeast-activated rat serum on a Bio-Gel P-60 column (see Materials and methods). Reconstituted lyophilized supernatant (350–400 mL) was loaded on a P-60 column (14.5 × 60 cm) equilibrated and eluted with 0.1 M ammonium formate at pH 5.0. All steps were carried out at 4 °C. Radiolabeled human ^{125}I-$C5a_{desArg}$ was added prior to gel filtration to monitor C5a elution during each step of the purification. Fractions of 35 mL were collected at a flow rate of 135 mL/h. Biologic activity and radioactivity were monitored, and C5a-containing fractions were pooled, dialyzed, and lyophilized for further purification. **B:** Ion-exchange chromatography of the C5a pool from Bio-Gel P-60 was performed on a QAE-Sephadex Q-50 column at 4 °C. The anaphylatoxin-containing pool was reconstituted in 1 mL 0.05 M NH_4HCO_3 at pH 8.6 and applied to a QAE-Sephadex Q-50 column (2.0 × 20 cm) equilibrated and developed with 0.05 M NH_4HCO_3 at pH 8.6. Fractions (2.5 mL) were collected at a flow rate of 10–20 mL/h. The anaphylatoxin-containing fractions were pooled as indicated by the horizontal bar. **C:** Rat $C5a_{desArg}$ obtained from the QAE-Sephadex column was chromatographed on a Pharmacia Mono-S column by FPLC at 4 °C. Solvent A is 0.1 M ammonium formate at pH 7.0 and solvent B is 0.8 M ammonium formate at pH 7.0. A linear gradient was developed from 100% solvent A to 27% solvent B for 5.4 min, and isocratic elution with 27% solvent B was continued for 25 additional min. Pool I indicates the $C5a_{desArg}$ fractions. Intact C5a elutes from the Mono-S column slightly later than the $C5a_{desArg}$ (chromatogram not shown).

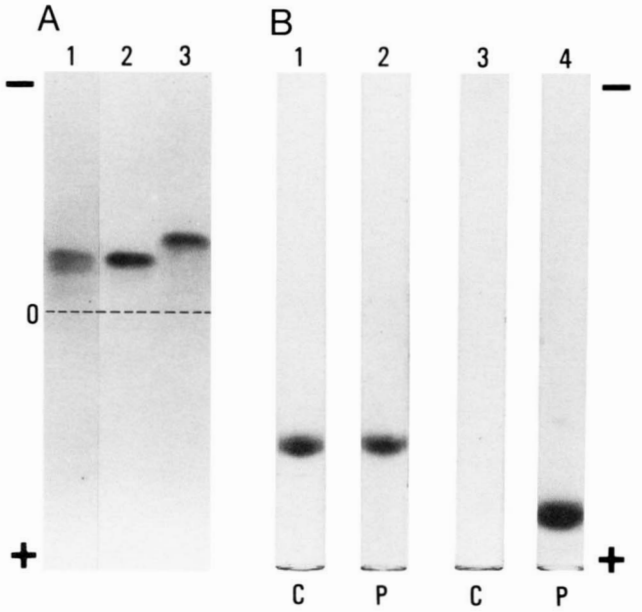

Fig. 2. A: Microzone electrophoresis on cellulose acetate strips in 0.075 M barbital buffer at pH 8.6 for 20 min. Lane 1, human C5a and trace of $C5a_{desArg}$; lane 2, rat $C5a_{desArg}$; lane 3, rat C5a. Protein bands were visualized by staining with amido black B10. The origin is noted by the symbol ○. **B:** Electrophoresis in SDS polyacrylamide gels (9%) containing rat C5a (gels 1 and 2) and human C3a (gels 3 and 4). Protein (P) was visualized by staining with Coomassie blue (gels 2 and 4) and carbohydrate (C) was visualized by staining with PAS reagent (gels 1 and 3). Note that rat C5a is a glycoprotein like human C5a (4) and the C3a control is devoid of oligosaccharide.

Table 1. *Amino acid composition of rat C5a and $C5a_{desArg}$*[a]

Amino acid	$C5a_{desArg}$ (residues/mol)	C5a (residues/mol)
Lysine	8.9 (9)	8.4 (9)
Arginine	6.1 (6)	6.9 (7)
Histidine	4.5 (5)	4.5 (5)
Aspartic acid	5.1 (5)	5.3 (5)
Threonine	2.7 (3)	2.8 (3)
Serine	1.2 (1)	1.5 (1)
Glutamic acid	11.2 (11)	11.0 (11)
Proline	2.6 (2)	2.8 (2)
Glycine	4.4 (4)	4.4 (4)
Alanine	6.2 (6)	5.9 (6)
Half-cystine	5.7 (6)[b]	6.0 (6)[c]
Valine	4.2 (4)	3.7 (4)
Methionine	1.1 (1)	1.0 (1)
Isoleucine	4.0 (4)	3.5 (4)
Leucine	5.3 (5)	5.1 (5)
Tyrosine	3.3 (3)	2.8 (3)
Phenylalanine	1.1 (1)	1.1 (1)
Total residues	(76)	(77)

[a] Residues in parentheses were taken from the primary structure analysis.
[b] Half-cystine was detected as a pyridylethyl-cysteine derivative.
[c] Six half-cystine residues were assumed from $C5a_{desArg}$ results.

toxins from various other species, contains 6 half-cystines detected here as pyridylethyl-cysteine. Analysis of rat C5a after a 5-min digestion with CpB showed that 1.06 mol of arginine was released per mol of C5a.

Amino acid sequence analysis of rat C5a

Primary structure analysis of rat C5a was obtained mainly from the PE-Cys derivative of $C5a_{desArg}$. A total of 60 residues from the NH_2-terminus of PE-Cys $C5a_{desArg}$ were identified by automated sequence analysis (see Table 2).

Compositional analysis of the peptide recovered in pool 13 after reverse-phase HPLC (C4 column) separation of a chymotryptic digest of PE-Cys $C5a_{desArg}$ (PE-CH13) indicated that peptide PE-CH13 contained both seryl and methionyl residues. Because neither serine nor methionine were identified in the first 60 residues, it was concluded that this fragment represented the C-terminal portion of the molecule. Primary structure analysis of fragment PE-CH13 is shown in Table 3. The linear arrangement of 19 residues was determined and 6 of the residues overlapped with positions 55–60 from analysis of the intact rat C5a (see Table 2). The 3 (lysyl) residues at position 55, 66, and 71 were not detected in the analysis of PE-CH13 due to modification during pyridylethylation or by later handling of the peptide. Therefore, carboxymethyl-Cys $C5a_{desArg}$ (CM-Cys $C5a_{desArg}$) was prepared, subjected to digestion with chymotrypsin, and the corresponding peptide was again isolated by RP-HPLC. Amino acid analysis of peptide 10 from the CM-Cys $C5a_{desArg}$ (CM-CH10) chymotryptic digestion mixture indicated that peptide CM-CH10 represents fragment 55–76 of rat $C5a_{desArg}$. The sequence of peptide CM-CH10 (Table 4) provided the lysine assignments at positions 55, 66, and 71 and confirmed the sequence obtained for PE-CH13. Asn-67 likely represents the site of glycosylation based on the known glycosylation attachment sequence Asn-X-Thr/Ser. A similar glycosylation site appears in human C5a at Asn-64 (Fernandez & Hugli, 1976, 1978).

The results from rat $C5a_{desArg}$ digested by CpY for different lengths of time and at various enzyme concentrations are given in Table 5. When the CpY digestion results for $C5a_{desArg}$ are combined with the CpB digestion results (1.06 Arg/mol C5a) from intact rat C5a, which released only arginine, a sequence of -Met-Leu-Leu-Gly-Arg was deduced for the C-terminal structure of rat C5a. The complete amino acid sequence proposed for rat C5a is presented in Figure 3. Based on an alignment that optimized homology between these 2 structures, residues 4–6 (Leu-Leu-His) in rat C5a appear to represent an insert when compared with the human C5a sequence (see Fig. 4).

Biological characterization of rat C5a ($C5a_{desArg}$)

The guinea pig is highly sensitive to the spasmogenic effects of C5a and is therefore the species of choice for evaluating spasmogenic activity and for comparing potency of C5a obtained from various sources. Based on the C5a-mediated contraction of guinea pig ileum, spasmogenic potency of rat C5a was estimated to be 25–30-fold greater than human C5a (Table 6). Removal of the C-terminal arginine from rat C5a reduced spasmogenic potency by only 3–4-fold, whereas human $C5a_{desArg}$ was 1,000-fold less potent than intact human C5a. In the guinea pig vascular permeability assay, rat $C5a_{desArg}$ was equal in po-

Table 2. *Primary structural analysis of PE-Cys C5a$_{desArg}$ determined by automated sequence analysis*

Cycle	Residue identified	Yield[a] (pmol)
1	Asp	1,980
2	Leu	2,112
3	Gln	1,594
4	Leu	2,205
5	Leu	2,078
6	His	718
7	Gln	1,717
8	Lys	1,490
9	Val	1,604
10	Glu	1,396
11	Glu	1,362
12	Gln	1,094
13	Ala	1,569
14	Ala	1,422
15	Lys	1,047
16	Tyr	1,101
17	Lys	965
18	His	615
19	Arg	826
20	Val	1,080
21	Pro	605
22	Lys	649
23	Lys	884
24	PE-Cys[b]	ND[c]
25	PE-Cys	ND
26	Tyr	623
27	Asp	777
28	Gly	357
29	Ala	574
30	Arg	396
31	Glu	432
32	Asn	360
33	Lys	345
34	Tyr	361
35	Glu	432
36	Thr	124
37	PE-Cys	ND
38	Glu	207
39	Gln	201
40	Arg	198
41	Val	200
42	Ala	234
43	Arg	164
44	Val	231
45	Thr	83
46	Ile	194
47	Gly	115
48	Pro	104
49	His	46
50	PE-Cys	ND
51	Ile	116
52	Arg	92
53	Ala	107
54	Phe	61
55	(X)[d]	ND
56	Glu	41
57	PE-Cys	ND
58	PE-Cys	ND
59	Thr	26
60	Ile	70

[a] Recoveries from 3,230 pmol of rat PE C5a$_{desArg}$.
[b] PE-cysteine was positively identified but not quantitated.
[c] ND, not done.
[d] X, residue not identified by sequence analysis.

Table 3. *Automated sequence analysis of C5a peptide PE-CH13*

Cycles	Assigned residue position	Amino acid identified	Recovery[a] (pmol)
1	55	(Lys)[b]	–
2	56	Glu	452
3	57	PE-Cys[c]	ND[d]
4	58	PE-Cys	ND
5	59	Thr	108
6	60	Ile	259
7	61	Ala	256
8	62	Asp	143
9	63	His	49
10	64	Ile	221
11	65	Arg	105
12	66	(Lys)	–
13	67	(Asn)[e]	ND
14	68	Glu	190
15	69	Ser	31
16	70	His	21
17	71	(Lys)	–
18	72	Gly	24
19	73	Met	9

[a] An aliquot of 750 pmol of peptide PE-CH13 was applied to the sequencer.
[b] Lysyl residues were modified by the pyridyethylation reaction.
[c] PE-cysteine was positively identified but not quantitated.
[d] ND, not done.
[e] Residue not identified by sequence analysis but assigned as Asn based on compositional analysis of PE-CH13. Asn 67 is the oligosaccharide attachment site in rat C5a.

tency to intact human C5a but 50-fold more potent than human C5a$_{desArg}$ (Fig. 5), demonstrating the potent vascular effect of rat C5a in guinea pig tissue.

To determine the relative affinity of rat C5a for a cellular C5a receptor, chemotaxis assays were performed using purified human neutrophils. Intact rat C5a stimulated chemotaxis with an ED_{50} of about 1 nM (Fig. 6), in agreement with the published ED_{50} for human C5a (Chenoweth & Hugli, 1980; Gerard et al., 1981). Interestingly, rat C5a$_{desArg}$ was nearly identical in chemo-

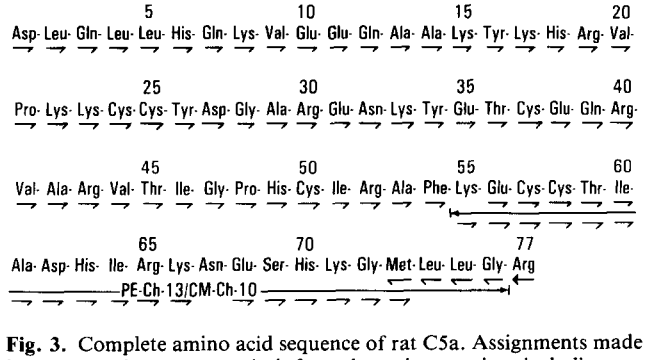

Fig. 3. Complete amino acid sequence of rat C5a. Assignments made by automated sequence analysis from the amino-terminus including residues 1–60 are combined with data from peptides PE-CH13/CM-CH10 (i.e., residues 55–73) as identified by the arrows (→). Assignments made by CpY digestion are identified by arrows (←). Assignments of Arg 77 made by CpB are denoted by an arrow (←).

Table 4. *Automated sequence analysis of C5a peptide CM-CH10*

Cycles	Assigned residue position	Amino acid identified	Recovery[a] (pmol)
1	55	Lys	284
2	56	Glu	219
3	57	CM-Cys	ND[b]
4	58	CM-Cys	ND
5	59	Thr	88
6	60	Ile	143
7	61	Ala	173
8	62	Asp	46
9	63	His	ND
10	64	Ile	133
11	65	Arg	55
12	66	Lys	93
13	67	(Asn)[c]	ND
14	68	Glu	84
15	69	Ser	31
16	70	His	197
17	71	Lys	52

[a] Recovery was calculated from 600 pmol of peptide CM-CH10.
[b] ND, not done.
[c] Residue not identified by sequence analysis, but assigned as Asn based on compositional analysis of PE-CH13.

tactic potency to intact rat C5a. In contrast, human $C5a_{desArg}$ and porcine $C5a_{desArg}$ (Chenoweth & Hugli, 1980; Gerard et al., 1981) are reportedly 10–100-fold less potent than the respective intact forms.

Biological characterization of the expressed LLH mutant of human rC5a

A major difference between the amino acid sequences of rat (or mouse) C5a and human C5a is an insertion of 3 residues (LLH) near the N-terminal end of rat C5a, located between Gln-3 and Lys-4 in the human sequence. To determine if this 3-residue insert contributed to the higher potency of rat C5a, a recombinant human C5a mutant containing the LLH insert was prepared. The sequence LLH was inserted into recombinant human C5a (human rC5a) between Gln-3 and Lys-4 by site-directed mutagenesis using a 30-mer oligonucleotide containing the mutated sequence, which was annealed to the corresponding region of the human C5a gene ligated into M13. Insertion of LLH in human rC5a failed to enhance potency of the mutant human C5a or $C5a_{desArg}$ when examined in 3 different functional assays (see Table 6; Figs. 5, 6).

Table 5. *Kinetics for carboxypeptidase Y digestion of rat $C5a_{desArg}$*[a]

Enzyme concentration (w/w): Digestion time (min):	1% 2	1% 7.5	2% 60	7% 60	10% 240
Gly	0.91	1.01	1.05	1.00	1.12
Leu	0.87	1.07	1.91	1.90	2.13
Met	–	–	–	0.22	1.13

[a] Reported as residues released on a mol/mol basis.

Discussion

It has long been appreciated that a factor derived from C-activated rat serum, later determined to be C5a, exhibited unusually potent anaphylatoxin activity (Friedberger, 1910; Friedberger et al., 1964). We have isolated rat C5a from C-activated rat serum for functional characterization and to evaluate the molecular basis for the higher potency ascribed to this protein in comparison with C5a derived from other species. Rat C5a consistently showed increased potency over human C5a by a factor of 5–25-fold using both tissue (guinea pig) and cellular (neutrophils) assays (see Table 6; Figs. 5, 6). More importantly, removal of the C-terminal arginine from rat C5a, by action of either serum-derived carboxyeptidase N or CpB to form $C5a_{desArg}$, resulted in a comparatively minor reduction in potency (see Table 6; Figs. 5, 6). In contrast, removal of the C-terminal arginine

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                          5         10        15        20        25        30        35        40
Rat      C5a  D L Q L L H Q K V E E Q A A K Y K H R V P K K C C Y D G A R E N K Y E T C E Q R

Mouse    C5a  N - H - - R - - I - - - - - - - - - S - - - - - - - - - - - V - F - - - - - E -
Porcine  C5a  M - -       K - I - - E - - - - - Y A M L - - - - - - - - - Y R - D D - - - - E -
Bovine   C5a  M - K       K - I - - E - - - - - R N A W V - - - - - - - - - H R - D D - - - - E -
Human    C5a  T - -       K - I - - I - - - - - - S - V - - - - - - - - - C V - N D - - - - - - -

                          45        50        55        60        65        70        75
Rat      C5a  V A R V T I G P H C I R A F K E C C T I A D H I R K N E S H K G M L L G R

Mouse    C5a  - - - - - - - - - L - - - - - - N - - - - - - - N K - - - E S P - - P V Q - - -
Porcine  C5a  A - - I K - - - K - V K - - - D - - Y - - N Q V - A E Q - - - N I Q - - -
Bovine   C5a  A - - I A - - - E - - K - - - S - - A - - S Q F - A D - H - - N I Q - - -
Human    C5a  A - - I S L - - R - - K - - T - - - V V - S Q L - A - I - - - D - Q - - -
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Fig. 4. Comparison of the rat C5a sequence with those of human, bovine, porcine, and murine C5a. The sequences were obtained from the following sources: human C5a, Fernandez and Hugli (1976, 1978); porcine C5a, Gerard and Hugli (1979, 1980); bovine C5a, Gennaro et al. (1986); and murine C5a, Wetsel et al. (1987).

Table 6. *Comparison of spasmogenic activity between rat and human C5a*[a]

Factor	Concentration range[b] (nanomolar)	Activity relative to rat C5a (%)
Rat C5a	0.005-0.03	100
Rat $C5a_{desArg}$	0.03-0.10	30
Human C5a	0.30-0.60	3.8
Human $C5a_{desArg}$	1,000-2,000	0.001
Human C5a mutant[c]	0.40-0.80	2.9
Human $C5a_{desArg}$ mutant	>1,000	<0.002

[a] Spasmogenic activity was obtained by measuring C5a-mediated contraction of guinea ileal strips (Cochrane & Müller-Eberhard, 1968)

[b] Minimum concentration of C5a required to elicit a full-scale ileal contraction.

[c] Human recombinant C5a mutant contains Leu-Leu-His inserted between residues Gln-3 and Lys-4 of the human C5a sequence.

from human C5a reduces potency by 10-1,000-fold in various biological assays (Hugli & Müller-Eberhard, 1978). These results suggest that the C-terminal arginine (Arg-77) in rat C5a is not as influential on effector function or high-affinity receptor interaction, as is Arg-74 in human C5a.

Primary structure comparisons (Fig. 4) revealed the presence of 3 additional residues (LLH) in the N-terminal region of rat C5a (LLR in mouse C5a) that are not present in human, porcine, or bovine C5a. In order to maximize sequence identity between the N-terminal regions of rat C5a and C5a from other species, the 3-residue LLH sequence was inserted between Gln-3 and Lys-4 of the human sequence. This 3-residue insert in the N-terminal region of rat C5a and mouse C5a (Wetsel et al., 1987) could play an important role in ligand-receptor interactions. The N-terminal region of C5a appears to contribute to ligand-receptor

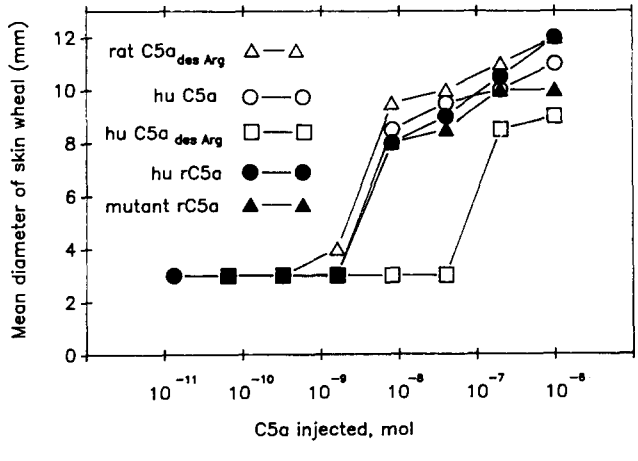

Fig. 5. The guinea pig vascular permeability assay was performed as described (Cochrane & Müller-Eberhard, 1968). In brief, 1 mL of Evans blue dye was injected intracardially. Fifty microliters of each dilution of C5a was injected subcutaneously. Thirty minutes later, permeability was assessed by removing the skin and measuring the area of blueing of the inside surface at each injection site. Rat $C5a_{desArg}$ was equal in potency to human C5a (natural and recombinant) as well as the recombinant human C5a mutant (LLH inserted between Gln-3 and Lys-4), whereas human $C5a_{desArg}$ was 50-fold less potent.

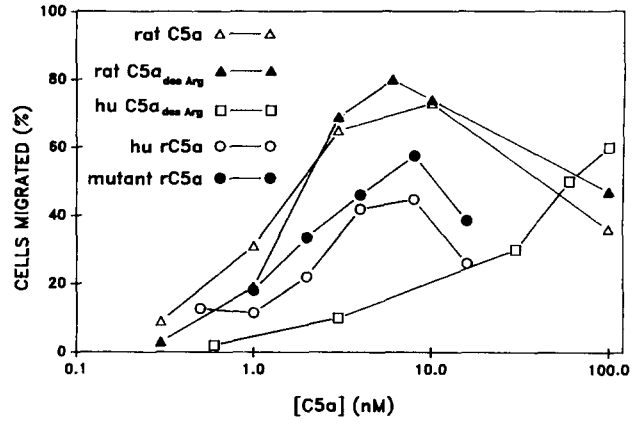

Fig. 6. C5a-mediated chemotaxis of human neutrophils was measured using a modified Boyden chamber assay (Keller et al., 1976). Human neutrophils (5×10^6/mL) were placed in the upper compartment of the apparatus. C5a at various dilutions was placed in the lower chambers. The relative number of neutrophils that migrated through an 8-μm pore size nitrocellulose membrane located between the 2 chambers was measured. ED_{50} values were obtained as follows: rat C5a (ED_{50} = 1.3 nM); rat $C5a_{desArg}$ (ED_{50} = 1.6 nM); human $C5a_{desArg}$ (ED_{50} = 40 nM); human recombinant (r) C5a (ED_{50} = 2 nM); and human rC5a mutant containing LLH inserted between Gln-3 and Lys-4 (ED_{50} = 1.5 nM).

interaction based on observations involving the N-terminal region of porcine (Gerard et al., 1985) and human C5a (Carney & Hugli, 1993). Modeling studies based on the NMR structure of human C5a (Zuiderweg et al., 1989), and later verified by site-directed mutagenesis studies (Carney & Hugli, 1993), suggested that specific residues in the N-terminal region (particularly Ile-6 and Tyr-13) contribute hydrophobic forces that stabilize the N-terminal region in the folded C5a structure. Therefore, we hypothesized that Leu-5 in rat C5a may be particularly important because this side chain could interact with neighboring hydrophobic residues in the disulfide-stabilized core region and/or the long C-terminal helix, if the N-terminal helix in rat C5a is oriented as it is in human C5a (Zuiderweg et al., 1989). However, insertion by site-directed mutagenesis of the LLH sequence at a corresponding location in recombinant human C5a (i.e., between residues Gln-3 and Lys-4) failed to significantly enhance the potency of either the recombinant C5a or $C5a_{desArg}$ mutants (see Table 6; Figs. 5, 6). These results suggested that the 3-residue extension in the N-terminal region of rat C5a contributes little to the enhanced potency observed for rat C5a.

Alignment of the primary structures of rat and human C5a revealed that 35% of the residues in these 2 sequences were nonidentical (Fig. 4). Fifteen of the nonhomologous residues in rat C5a differ substantially in side-chain character in comparison to the corresponding residues in human C5a. Although these 15 residues were distributed throughout the C5a molecule, half were located in the C-terminal helical region (Fig. 7). Sequence differences in the C-terminal region are of special interest because this region contains the primary C5a effector site (Chenoweth & Hugli, 1980). Recent evidence has shown that a 21-residue C-terminal peptide corresponding to rat C5a 57-77 is 10-fold more potent than the human counterpart (i.e., human C5a 54-74) in a spasmogenic assay (Ember et al., 1993). Three charged or polar residues in the C-terminal region of rat C5a were substituted for hydrophobic residues in human C5a (Thr-

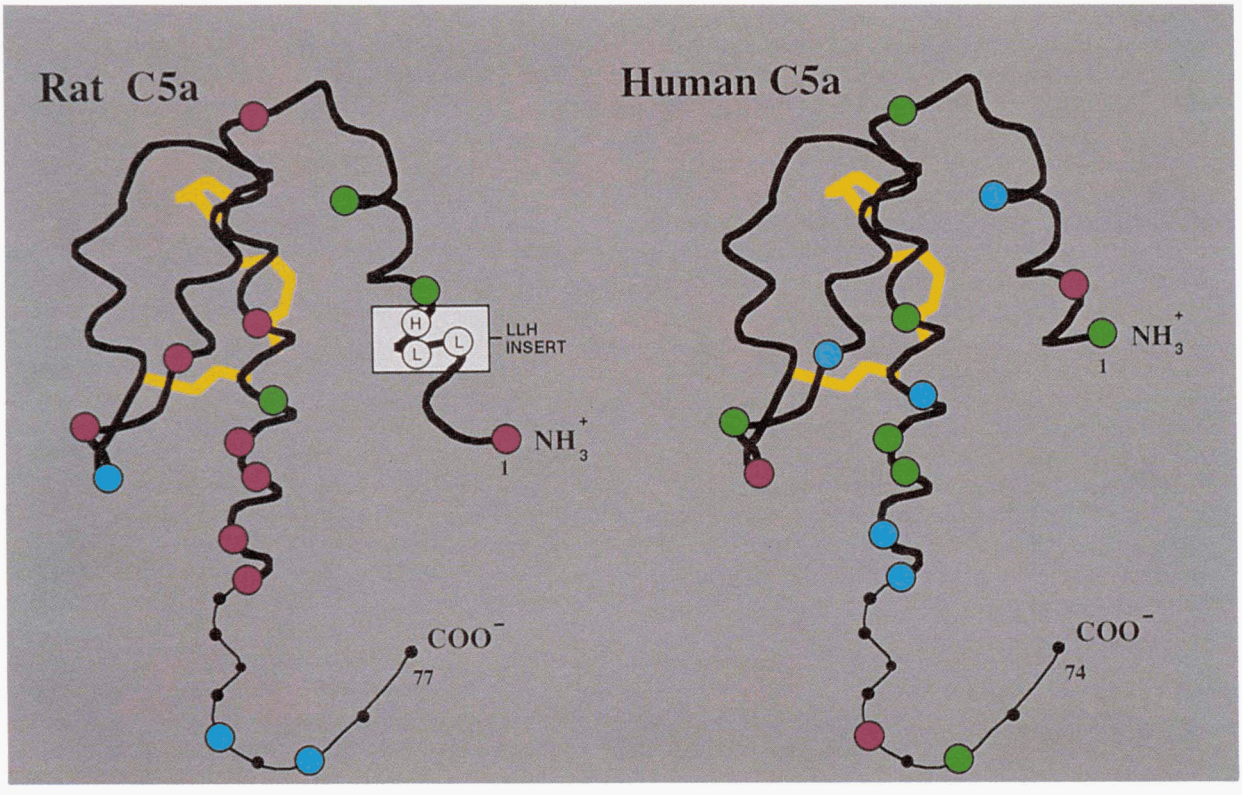

Fig. 7. Nonhomologous residues that differ in side-chain character between rat and human C5a are shown at their relative positions along the backbone structure of C5a. The colors indicate residue side chains that are polar and charged (red), polar and uncharged (green), or hydrophobic (blue). The yellow lines indicate disulfide bonds. The backbone structure was adapted from the NMR solution structure for porcine C5a (Williamson & Madison, 1990) and represents residues 1–65. Because the structures of porcine and human C5a (Zuiderweg et al., 1990) are undefined in solution after position 65, the backbone beyond residues 68 in rat C5a or 65 in human C5a are arbitrarily indicated as a thin line.

59 → Val, Lys-66 → Ala, and Glu-68 → Ile), and 2 charged residues in rat C5a were substituted for 2 polar residues in human C5a (Asp-62 → Ser, His-63 → Gln). Interestingly, the only 2 nonpolar residues in this region of rat C5a that are substituted for polar residues in human C5a (Gly-72 → Asp and Leu-74 → Gln) were located in the C-terminal effector region.

Extensive site-directed mutagenesis studies have been performed with human C5a (Zuiderweg et al., 1989; Carney & Hugli, 1993; Toth et al., 1994) and these results could prove useful in delineating substituted residues in rat C5a that contribute to the higher potency of this factor. Site-directed studies have demonstrated that the N-terminal region probably does not directly interact with the C5a receptor (Zuiderweg et al., 1989; Carney & Hugli, 1993), and thus sequence differences in this region of rat C5a compared to other species should not contribute to its higher potency. In contrast, a number of residues in the C-terminal region of human C5a, including Ser-66, His-67, Lys-68, Met-70, Leu-72, Gly-73, and Arg-74 have been shown to have significant effects on receptor interaction when substituted by site-directed mutagenesis (Zuiderweg et al., 1989; Toth et al., 1994). Both conservative and nonconservative substitutions of Ile-65, Asp-69, and Gln-71 failed to significantly affect receptor interactions (Zuiderweg et al., 1989; Toth et al., 1994), thus the corresponding nonhomologous residues Glu-68, Gly-72, and Leu-74 in rat C5a may not contribute to function in a major way. Interestingly, substitution of Arg-46 in human C5a for Ala, His, or Lys showed significant changes in C5a potency (Toth et al., 1994), indicating that the corresponding residues in rat C5a (namely His-49) are a candidate for site-directed mutagenesis studies. Unfortunately, substitutions between residues Asn-30 to Gln-60 in human C5a have not been performed. Thus, nonhomologous residues within this region in rat C5a, namely, Lys-33, Tyr-34, Lys-55, Thr-59, His-63, and Lys-66, all require further examination by site-directed mutagenesis to assign contributions by these residues to receptor binding of the ligand.

It is likely that variability observed in the potency for rat and human C5a, using various biological assays, also reflected differences at the receptor-binding level. The complete amino acid sequence for C5a receptors derived from human, mouse, and dog C5a and partial sequences derived from rat and bovine C5a receptors have revealed approximately 70% sequence identity among these receptors (Boulay et al., 1991; Gerard & Gerard, 1991; Gerard et al., 1992; Perret et al., 1992). C5a receptors belong to the rhodopsin family of G protein-coupled receptors that consists of 7 transmembrane-spanning domains. Interestingly, the greatest divergence in sequence identity among the various species exists in the nontransmembrane external loops. To what extent sequence variations in these receptor regions contribute to differences in the binding of C5a from different species is not yet known.

Another matter of interest is the target tissue (or cells) used in biological assays for C5a. Our finding that the potencies of

rat $C5a_{desArg}$ and human $C5a_{desArg}$ differ markedly in the guinea pig ileal assay, yet are substantially less divergent in the guinea pig vascular permeability assay (i.e., 20,000-fold versus 50-fold, see Fig. 6; Table 6), reflects differences with respect to tissue specificity. C5a-mediated smooth muscle contraction is a histamine-dependent event (Hugli & Müller-Eberhard, 1978), although it is not yet clear whether C5a stimulates histamine release from mast cells directly or stimulates other C5a receptor-bearing cells such as tissue macrophages or fibroblasts, which then release mediators that stimulate mast cell histamine release. In any case, it is apparent that cells in guinea pig ileum contain C5a receptors with unusually high affinity for rat $C5a_{desArg}$. It has been demonstrated that the C5a receptor is derived from a single copy gene (Gerard et al., 1992; Perret et al., 1992), thus arguing against the existence of multiple C5a receptor isotypes of varying affinity on different cell types within a species. Alternatively, cell type-specific cellular factors that influence ligand affinity and/or the activation state of rhodopsin family receptors including G protein-receptor interactions, signal messengers, and receptor kinases (Koo et al., 1982; Didsbury et al., 1991) could in part account for differences observed in apparent C5a potency observed in various biological assays.

In summary, the primary structure and biological characterization of rat C5a are presented. Rat C5a was shown to be significantly more potent than human C5a in a variety of biological assays, especially when the desArg forms of these 2 molecules were compared. Sequence comparisons between rat C5a and C5a from other species revealed a number of residue substitutions throughout the molecules, including an insert of 3 residues near the N-terminus of rat C5a. Site-directed mutagenesis data suggested that the N-terminal extension does not contribute to the enhanced potency of rat C5a.

Materials and methods

Chemicals and materials

Bio-Gel P-60 and QAE-Sephadex Q50-120 were obtained from Bio-Rad (Richmond, California) and Sigma (St. Louis, Missouri), respectively. The Mono-S (HR 5/5) column was purchased from Pharmacia (Uppsala, Sweden). Pooled rat serum was obtained from Biotrol Company (Indianapolis, Indiana). All other reagents and solvents were of the highest chemical purity obtainable.

Bioassays

Spasmogenic activity of anaphylatoxins was measured using terminal strips of guinea pig ileum as described by Cochrane and Müller-Eberhard (1968). Neutrophil chemotactic activity of the anaphylatoxins was measured by a modification of the procedure of Keller et al. (1976) as described by Dahinden et al. (1984). The guinea pig vascular permeability assay was performed as previously described (Cochrane & Müller-Eberhard, 1968).

Mutagenesis

To investigate the contribution of the 3-residue extension (LLH) in the N-terminal region of rat C5a, the codons for these 3 residues were inserted into recombinant human C5a between Gln-3 and Lys-4 by standard mutagenesis techniques. The mutated human C5a cDNA was ligated into the pMAL-p expression vector (New England Biolabs). The recombinant human C5a mutant was expressed in *Escherichia coli* as previously described (Carney & Hugli, 1993).

Purification of rat $C5a_{desArg}$ and rat C5a

Two liters of serum from normal adult rats (4 strains) were activated by adding zymosan to a final concentration of 20 g/L of serum and incubating at 37 °C for 45 min (David & Reisfeld, 1974). The mixture was acidified by adding concentrated HCl to a final concentration of 1 N HCl and then neutralized to pH 4.5 with NH_4OH. The acidified serum contained a heavy precipitate that was centrifuged and the supernatant was transferred to small pore tubing (3,000 M_r cutoff) and dialyzed against 4 changes of water (15 L each) and then lyophilized.

The lyophilized powder was reconstituted in 350–400 mL of water and was applied to a Bio-Gel P-60 column (14.5 × 60 cm) and eluted with 0.05 M ammonium formate at pH 5.0. Radiolabeled human $C5a_{desArg}$ (10^7 cpm), iodinated by the lactoperoxidase method (David & Reisfeld, 1974), was added prior to gel filtration as a means of monitoring the elution of rat C5a or $C5a_{desArg}$. The fractions that contain anaphylatoxin as judged by either radioactivity or biologic activity were pooled and further fractionated on a QAE-Sephadex Q-50 column that was equilibrated and developed with 0.05 M NH_4HCO_3 at pH 8.6. The fractions containing anaphylatoxin activity were pooled and applied to a Mono-S column for the final step of purification. A gradient from 100% buffer A (0.1 M ammonium formate at pH 7.0) to 27% buffer B (0.8 M ammonium formate at pH 7.0) was developed over 5.4 min at a flow of 1 mL/min, and isocratic elution was continued with 27% buffer B for an additional 35 min to affect elution of rat $C5a_{desArg}$.

Preparation of rat C5a followed a similar isolation procedure to that described above except that a carboxypeptidase inhibitor, DL-2-mercaptomethyl-3-quanidinoethylthiopropanoic acid, was added to the serum prior to zymosan activation (Hugli et al., 1981). The rat C5a elutes at a position just after $C5a_{desArg}$ during isocratic elution on the Mono-S column (see Fig. 1).

Homogeneity of the rat C5a and $C5a_{desArg}$ was evaluated by electrophoresis on cellulose acetate strips (Beckman Microzone) and in SDS-polyacrylamide gels (SDS-PAGE). The former procedure was carried out in a 0.075 M barbital buffer at pH 8.6 using a Beckman model R101 electrophoresis apparatus operated at 220 V for 30 min under room temperature conditions. The SDS-PAGE was performed in 0.1% SDS using 9% gels at pH 7.2 (Weber & Osborn, 1969).

Amino acid analysis

Amino acid analyses were performed on rat C5a, $C5a_{desArg}$, fPE-Cys $C5a_{desArg}$, and CM-Cys $C5a_{desArg}$. Samples were hydrolyzed for 24 h in 5.7 N HCl containing 1% (v/v) phenol at 110 °C. Amino acid analyses were carried out on a Beckman amino acid analyzer, model 121M. PE-Cys $C5a_{desArg}$ was prepared according to the method described by Friedman et al. (1970). Reduction and carboxymethylation of $C5a_{desArg}$ was carried out according to the procedure of Crestfield et al. (1963). Chymotrypsin digestion was performed in 0.1 M NH_4HCO_3, 0.1 M $CaCl_2$ at pH 8.0–8.5 using 1% enzyme (w/w). Rat C5a

fragments from the chymotrypsin digestion mixture were separated by HPLC methodology on a Vydac 5 C4 reverse-phase column, and the conditions used are presented in Figure 2. Amino acid compositions of the chymotryptic C5a fragments were obtained using a Waters Pico Tag analysis system and a Beckman 6300 autoanalyzer.

Primary structural analysis of rat C5a

Amino acid sequence analyses of rat C5a were performed with the aid of an Applied Biosystems model 470A autosequenator. The amino acid derivatives were analyzed by HPLC methodology using an Altex Ultraspheres ODS column. Determination of C-terminal amino acids in C5a and $C5a_{desArg}$ was accomplished by digestion with 1.5% (w/w) CpB in 1% $NaHCO_3$ at pH 8.0–8.5 and 37 °C for 5 min and with 1, 2, 7, and 10% CpY in 0.05 M sodium acetate at pH 5.5 and 37 °C for 2–240 min. Released amino acid residues were analyzed using a Beckman amino acid analyzer, model 121M.

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